

Title        Studies on Cryopreservation of Early  
Stage Zebrafish (Dan/O Rer/O) Oocytes  
Using Controlled Slow Cooling

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## ABSTRACT

In the last three decades, much effort has been made toward the successful cryopreservation of fish gametes. The spermatozoa from many species including salmonid, cyprinids, and acipenseridae has been successfully cryopreserved and well documented. Systematic studies on fish oocytes cryopreservation has not been carried out until recently. In this study, the effect of cryoprotectant toxicity on stage I and II zebrafish oocytes were studied and the controlled slow cooling was used at different cooling rates to identify the optimal cooling rate for these oocytes and the optimal conditions for removing cryoprotectant. Methanol was found to be the least toxic CPA for zebrafish oocytes and 4M methanol in potassium chloride (KCl) buffer was used as the cryoprotective solution. Oocytes were cooled with programmable cooler (Planner KRYO 550) using controlled slow cooling at 0.3, 0.5 and 1°C/min rates, combined with seeding at -12.5°C and plunge into liquid nitrogen at -80°C. 1°C/min was identified to be the optimal condition for cryopreservation of stage I and II oocytes. High survival rate were obtained after cooling to -196°C assessed with TB staining which were  $87.8 \pm 4.9\%$  and  $86.8 \pm 6.5\%$  for stage I and II respectively. It was also found in this study that recovery conditions affect oocyte survivals after freezing. Oocytes which had been incubated for 60 or 120min in room temperature showed higher survival rate than those assessed immediately after freezing. This study has shown that stage II (cortical alveoli stage) oocytes were more resistible to freezing than stage I (primary growth stage) oocytes. More oocytes viability studies are needed to validate these results.

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# 1.0 INTRODUCTION

## 1.1 Principles of cryobiology

Cryobiology was defined by Sir Alan Parkes in the journal *Cryobiology* (Parkes, 1964) as the study of frosty life. This technology deals with solving practical problems in health care, biosciences and conservation. Cryobiology is concerned with the state of water in cells, role of water in the structure and function of cell components and the factors affecting the transformation of water into ice. Although freezing cannot remove the 10% residual water of hydrated cells, that fraction can be removed by freeze-drying or by air, vacuum drying from the liquid state. The major factor that determines whether or not cells survive freezing to low subzero temperature is the rate at which they are cooled. Cells are at normal isotonic volume before freezing, when they are cooled slowly. They are shrunken before freezing meaning that the higher the osmolality of the external medium, the greater the unfrozen fraction, the lower the probability that the cell plasma membrane will come in direct contact with ice (Rall *et al.*, 1984).

Cells are surrounded by lipid bilayer membranes. These membranes serve to separate and protect a cell from its surrounding environment and it is well known that certain proteins and peptides are able to create channels and in this way selectively regulate the permeability and stability of membranes, the water molecules that hydrate the polar surface have to be pushed aside



(Leikin *et al*, 1993). The movement of water and solute occurs through the plasma membrane, and the rate of movement is determined by its structure and composition (Davson and Danielli, 1952). Additional water channel proteins have been found in a broad range of different mammalian cells and tissues (Edashige *et al.*, 2000, Sui *et al.*, 2001) and others found in insects and plants.

Cryobiology studies the physicochemical and biophysical changes which occur during freezing and thawing, as well as the importance of post-thaw processing of the tissue, which can affect the tissue. Cryobiology tries to explain the damaging factors in high and low cooling rates and the conditions leading to optimum cooling rate varying over a broad range in different cell types (Whittingham *et al.*, 1972). Cryoprotectants protect slowly cooled cells in proportion to their concentration but they confer no protection to cells cooled at super optimal rates (Wellman & Pendyala, 1979). Cryoprotectant act primarily by reducing the amount of ice that is formed at any given subzero temperature. In some cases if cryoprotectant could be introduced sufficiently, freezing would be avoided altogether and a glassy or vitreous state could be produced, but osmotic and toxic damage caused by the high concentrations of cryoprotectant that are required then become critical problems. The transport of cryoprotectants into and out of cells and tissues is sufficiently well understood to make optimization by calculating a practical possibility, but direct experiment remains crucial to the development of other aspects of the cryopreservation process. Cells that do not contain cryoprotective solute or additives tend to undergo intracellular ice formation (Diller, 1979). This is

because CPAs generally reduce the hydraulic conductivity ( $L_p$ ) at all temperatures therefore increasing viscosity and also because they reduce the freezing point of the cytoplasm (Myers *et al*, 1994).

### 1.1.1 Chilling injury

Injury occurs during freezing when cells are exposed to increasing concentration of solutes or by the formation of intracellular ice. Chilling injury is injury associated with low temperature without the formation of ice. Cooling reduces viability even before freezing temperatures are reached.

Chilling injury may be distinguished from injury resulting from high cooling rate which is probably due to osmotic damage. The term chilling injury was originally used in the botanical world as early as 18th century to describe the phenomenon that plants subjected to chilling temperature above 0°C were often damaged irreversibly (Levitt, 1980). Lipids in cell membranes would be expected to undergo a sol -to-gel phase transition in a range between 0°C and -20°C, the temperature range of maximum chilling injury. Effects of cooling are classified in two categories (Morris and Waston, 1984): direct chilling injury or cold shock and indirect chilling injury. These two categories are components of the same phenomenon but differ in response to different types of cells. They can be quantitative rather than qualitative (Pegg *et al*; 1990).

Direct chilling injury or cold shock affects cells when cooled rapidly enough to a sufficiently low temperature. The response of any cell-type maybe modified by the culture conditions before cooling or by the addition of specific compounds. Cellular viability depends on the rate of cooling with more injury occurring during rapid cooling and also loss of membrane permeability. Direct chilling injury is almost independent of the rate of warming. Injury is increased as the period incubation at the reduced temperature is extended (Pegg, 1990). Lipid phase transitions in the cell membrane have been confirmed to be responsible for cold shock injury in sperm of many species, mammalian, human platelets and fish oocytes (Pearl *et al*; 2000).

Indirect chilling injury is observed when there is a relatively long exposure period at the reduced temperature. It is independent of the rate of cooling. Indirect chilling injury may manifest rapidly in mammalian oocytes or embryos. Chilling injury is used to refer to the damage following exposure to low temperature without freezing and freezing damage. Low temperature may affect the structure and function of protein by decreasing the rate of enzyme activity (Morris & Clarke, 1987). Studies with *Drosophila* embryo have shown that chilling injury in permeabilised embryos containing ethylene glycol was roughly comparable to that in intact embryos which contained cryoprotectants (Zhang & Rawson, 1995). Chilling injury has been suggested to be associated with lipid membrane phase transition, depolymerisation of microtubules, disruption of cell division and denation of protein (Arav *et al*; 2000).

Zebrafish oocytes are chilling sensitivity and chilling injury is linked to the membrane lipid phase transition (Pearl & Arav, 2000).

### 1.1.2 Freezing injury

The cells and their surrounding medium remain unfrozen when the temperature is down to about -5°C because of supercooling, in some medium because of salt concentration and the depression of the freezing point by the protective solutes that are frequently present (Mazur *et al.*, 1981).

There is an increase in solute concentration in the extracellular solution as the temperature decreases, due to extracellular ice formation, resulting to an imbalance in a chemical potential between the biomaterial and the exposure and the unfrozen external solution (Acker *et al.*, 2001). When cooling is too rapid, the rate at which chemical potential of water in the extracellular solution decreases is much faster than the rate at which water can diffuse out of the cell (Muldrew & McGann, 1994) and this results to an intracellular ice formation but if cooling is adequately slow, the cell loses water rapidly by exosmosis to concentrate the intracellular solutes sufficiently to eliminate supercooling and maintains the chemical potential of intracellular water in equilibrium with that of extracellular water resulting to cell dehydration (Pitt & Steponkus, 1989).

During freezing and thawing cells are either damaged or survive due to bulk water transport across cellular membrane (Farrent *et al.*, 1977). Intracellular ice formation (IIF) during freezing damages cells, some survive intracellular

freezing provided that thawing is extremely rapid, over the temperature range of -30 to 0°C (Acker *et al.*, 1999). However some Antarctic species have been reported to survive intracellular freezing as intact organisms. Intracellular ice formation occurs in three possible ways. The homogeneous nucleation: temperature of a 1 µm droplet of pure water is -39°C, and increase by approximately 2°C for each 10-fold increase in droplet diameter. Solutes depress the homogeneous nucleation temperature by 3.3°C for each unit increase in solution osmolality. The expected range of homogeneous nucleation temperature is -38°C to -44°C. Heterogeneous nucleation depends on the presence of intracellular nucleating agents. Intracellular ice forms at -31 to -38°C in cells cooled in micro-droplets (Franks *et al.*, 1983). Seeding by the extracellular ice: Intracellular ice forms at a median temperature of -10 to -20°C in many cell types showing that seeding is used to prevent IIF in many cases by introducing ice nuclear element. At low cooling rates, freezing propagates extracellularly, the solute concentration outside the cell begins to rise, causing osmotic dehydration of the cells (Bischof and Rubinsky, 1993). Solutes become concentrated within the cells, the concentration of solute injures the cell by damaging enzymatic mechanism (Lovelock, 1953) and destabilizes the cell membrane (Steponkus, 1984). Intracellular ice formation (IIF) occurs when the cooling rate is sufficiently rapid to trap water within the cell and the cell cannot osmotically equilibrate with the extracellular space. In this case, the cytoplasm cools and ice ultimately nucleates within the cell (Mazur, 1965); and these ice crystals cause injury to the organelles and membranes (Toner *et al.*, 1990). It is an implicit assumption that the formation of ice inside the cell is inevitably lethal (Mazur, 2004). Ice first forms in the

external medium, it removes pure water from the solution and causes the concentration of extracellular solutes to rise.

Lovelock (1953) proposed that high salt concentration during freezing was important, and indirectly responsible for damage linked to high extracellular osmolarity causing cells to be desiccated beyond their limit, shrunken below their “minimum cell volume,” and thus the cells were destroyed. Solution effects are due to the high salt concentration, the physical relationship between the cells and the surrounding ice matrix, pH fluctuations with temperature and concentration of buffers. Although other aspect of the events has not been satisfactorily demonstrated (Watson & Fuller, 2001).

Mechanical effects of extracellular ice crystals at cell surfaces especially in tissues with cellular interconnections and alternations in the physical properties of solutions outside the cell, including the concentration of solutes. These results from the nucleation of a proportion of extracellular water and can also be damaging (Muldrew and McGann, 1990). It was suggested that cells are damaged by the mechanical interaction between the growing ice phase and cells sequestered between ice crystals causing haemolysis in erythrocytes (Pegg and Diaper 1989).

### 1.1.3 Thawing injury

Thawing is an important stage in cryopreservation, during thawing many processes may occur, which may positively or negatively affect the viability of cryopreserved cells. Frozen cells are thawed before their viability is assessed. Slow thawing or fast thawing is usually conducted in a programmable cooler, or by exposing the sample to the air at room temperature. Fast thawing is normally conducted through immersing the samples into a water bath with a set temperature (20°C-38°C). Thawing of cells in water or air has certain limitations, including uneven warming of the samples and the lack of controls over the process. Cells can be damaged due to recrystallization during warming and innocuous intracellular ice crystals in rapidly cooled cells will grow if warming is too slow (Rall *et al.*, 1984). Some cells with intracellular ice can survive if thawed very rapidly because rapid thawing generally improves survival (Karow and Pegg, 1981).

Most cryobiologists considered the fast thawing more advantageous as it lessens the risk of recrystallisation and formation of large ice crystals inside the sample. It also shortens the time of exposure of cells to CPAs at above eutectic temperature (Gordienko & Pushkar, 1994).

Intracellular ice formation (IIF) is favoured when cells are cooled in rate more than the optimal cooling rate. They are also sensitive to a decrease in the rate of thawing. *Cyprinus carpio* embryos showed that the optimum survival of these embryos required warming at a slow rate (8°C/min), since fast thawing rate killed the embryos (Zhang *et al.*, 1989). Injury caused by rapid thawing is

due to the presence of a small amount of intracellular ice which results in osmotically induced damaging water movements during the initial part of thawing (Ashwood-Smith; 1980 and Farant; 1980). Mechanical stresses can affect and damage cell in the course of rapid heating of a frozen biological system due to the limited conductivity of the system. At low temperatures a two step warming combination of slow and rapid warming is better in order to improve survival (Vorotilin *et al*, 1991).

#### **1.1.4 Cryoprotectants**

Cryoprotectants (CPAs) are substances characterised by their ability to reduce cryoinjury of biological materials during the course of freezing. Insects most often use sugars as cryoprotectants. Arctic frogs use glucose, but arctic salamanders create glycerol in their livers for use as cryoprotectant. There are about one hundred substances which have been applied as cryoprotective agents. Cryoprotectants prevent actual freezing and the solution maintains some flexibility in a glassy phase (Buitink *et al*, 1998). Natural systems that survive extreme environmental stress have shown that one of the adaptive mechanisms used is the overproduction and accumulation of sugar (Crowe and Crowe, 2000). In the absence of more traditional cryoprotectants e.g DMSO and glycerol, sugars have been shown to be effective protectants in mammalian cell cryopreservation (Eroglu *et al* ; 2000).



Cryoprotectants are classified into two categories, the permeating and non-permeating cryoprotectants. Permeating cryoprotectants are cryoprotective substance which penetrates inside the cells and conduct their protective action inside the organelles and cytoplasm e.g methanol, dimethylsulfoxide (DMSO), glycerol and propylene glycol (PG), ethylene glycol (EG). These cryoprotectant are low molecular weight chemicals and can penetrate inside the cell membrane and tissues, and act as ice formation suppressors, osmotic buffers and membranotropic agents. The non-permeating cryoprotectants e.g hydroxethyl starch, polyvinyl pyrrolidone and various sugars, which are high molecular weight agents. Sugars especially disaccharides are often added to cryoprotective media in combination with intracellular CPAs and conduct their protective action through osmotic dehydration of cells therefore lessening the risk of intracellular ice formation (Fahy, 1986, Pegg and Arnaud, 1988, Fahy *et al*, 1990).

DMSO is most widely used cryoprotectant. It has been used in cryopreservation of cell types, tissues and organs. PG and EG have been widely used for vitrification of mammals oocytes and ovarian tissue, and also in controlled slow cooling programmes. Sucrose is widely used as an extracellular cryoprotectant. Methanol have been shown to be the most effective cryoprotectant for zebrafish embryo and sperm of several fish species (Plachinta *et al*, 2004). Mixtures of cryoprotectants may have less toxicity and are more effective than single agent cryoprotectant. Cryoprotectant mixtures have been used for vitrification i.e, solidification without ice formation (Pegg and Arnaud 1988).

Water and cryoprotectant permeability of the membranes of cells and tissues is important not only for the addition and removal of CPAs but also because the fundamental physical mechanisms of solute and water transport across a cell membrane, e.g. channels versus lipid bilayer transport can be characterized (Crowe *et al*, 1989). However the recent discovery and characterization of water channels in biological membranes reveals that aquaporins are highly selective for water and do not typically co-transport cryoprotectants.

#### **1.1.5 Approaches used in cryopreservation**

Cryopreservation is a process where cells, tissue and organisms can be preserved by cooling to low sub-zero temperatures for a long period.

These viable cells are sustained using liquid nitrogen at or close to, -196°C. At these low temperatures any biological activity is effectively stopped and normal cellular chemical reactions do not occur, as kinetic energy levels are too low to allow the necessary molecular motion (Grout *et al*, 1990).

A limited success has been reported on the cryopreservation of embryos of aquatic species and eggs including the oocytes and embryos of specific oyster (Lin & Chao, 2000; Smith *et al*, 2001; Tervit *et al*, 2005). Although sperm and larvae have been cryopreserved effectively, the successful cryopreservation of oocytes and fertilized egg still remains elusive (Adams, 2003). Successful cryopreservation was achieved by New Zealand group of

researchers. Pacific oyster (*crassostrea gigas*) eggs have been cryopreserved in liquid nitrogen temperature with up to 25% post-thaw fertilization rate (Smith *et al.*; 2001) and even enhanced in more recent studies, where up to 50% of oyster oocytes survival was attained (0.3°C/min) slow cooling and 10% ethylene glycol was used as cryoprotectant (Tervit *et al.*, 2005).

Two approaches have been used to cryopreserve biological materials: controlled slow cooling and vitrification.

#### **1.1.5.1 Controlled slow cooling**

Cryopreservation attempts to design a cooling protocol that takes the cells through the period of vulnerability with least damage. This is known as controlled slow cooling. There are several factors to take into consideration in this process which are the cryoprotectant type, exposure time and temperature, cooling rate, seeding and thawing conditions.

During slow cooling cells maintain an osmotic equilibrium with the extracellular solution through dehydration. When this happens intracellular ice may be avoided, although damage can still occur when there is solute toxicity (Lovelock, 1953, Mazur *et al.*; 1972) and physical changes to the cell induced by excessive cell shrinking under an osmotic stress (Steponkus and Wiest, 1978).

During cooling from 0°C to -20°C ice crystals start to form in the bathing medium, increasing the solute concentration. As a result water

moves out from cells down to a concentration gradient causing cell shrinkage and dehydration (Lovelock, 1953). In order to maintain a control rate of cooling, super cooling of the bathing medium must be avoided. Basic steps taken in controlled slow cooling approaches are:

- i Collection and assessing of cell quality
- ii Equilibrating cell in cryoprotectant,
- iii Ice seeding
- iv Freezing cells using controlled slow cooling to below -80°C,
- v Plunging and lowering temperature storage at -196 °C,
- vi Warming and thawing cells using controlled conditions
- vii Removing cryoprotectant
- viii Return cells to normal physiological conditions.

In controlled slow cooling, cryoprotectant additives (CPAs) has three main functions; they assist in the dehydration process before external ice formation. They may have a protective effect on cellular structures (e.g membranes) reducing salt effects. They reduce the water activity levels in the absence of total dehydration (Steponkus and Wiest 1978). Controlled slow cooling was used in the study of cryopreservation of zebrafish (*Danio rerio*) oocytes by Plachinta *et al*, (2004, 2005). Cryoprotectant solutions were prepared in Hank's medium. 2M PG, DMSO, methanol and 1M EG were used in the study. Oocytes were incubated in CPA solutions for 30min at room temperature before frozen with different cooling rates (1 or 2°C/min) to -10, -5, -20 or -25°C. two viability assessment methods were applied in the study: trypan blue (TB) staining and germinal vesicle breakdown (GVBD)

observation. Results showed that oocytes viability decreased with temperature and methanol seems to be the most effective cryoprotectant. Another studies using controlled slow cooling on membrane integrity and cathepsin activity of zebrafish (*Danio rerio*) oocytes after freezing at  $-196^{\circ}\text{C}$  were carried out by Zhang et al 2005 using 2M methanol and 2M DMSO as cryoprotectants. After cooling membrane integrity and cathepsin activities of oocytes were assessed both after cryoprotectant treatment at  $22^{\circ}\text{C}$  and after freezing in liquid nitrogen. DMSO appeared to be better cryoprotectant than methanol. Fish embryo cryopreservation using controlled slow cooling have indicated that although embryos from all species show a certain degree of survival at subzero temperatures, they do not normally survive after cooling to  $-30^{\circ}\text{C}$  (Zhang *et al*; 1993).

#### **1.1.5.2 Vittrification**

Vitrification is one of the approaches used for cryopreservation of cells and organs at extremely low temperature without freezing. It is the process of converting a material into a glass-like amorphous solid, free of any crystalline structure. However the term is now used more generally to refer to a process in which attempts are made to vitrify the whole system to avoid any ice formation (Armitage and Rich, 1990 Fahy 1989, Pegg and Diaper, 1990). If a liquid is cooled fast enough, then the viscosity might increase to a level where molecular translation is too slow to allow

crystal growth or nucleation from occurring (Fahy *et al.*, 1984). Vitrification has been used to cryopreserve human 4- and 8-cell embryos (Mukaida *et al.*; 1998); 40% ethylene glycol together with 18% Ficoll and 0.3 mol/l sucrose were used as a low toxicity solution with stable vitrification properties. Mixture of water and high concentrations of many water soluble chemicals can vitrify. Vitrification prevents damage associated with ice formation, cellular osmotic dehydration and shrinkage during slow freezing, intracellular ice formation and destructive intracellular ice re-crystallization during rapid freezing and during slow freezing, mechanical disruption of extra-cellular structure in organised tissues and organs (Bodziony *et al.*; 1994, Rall and Fahy, 1985). When materials are vitrified, no ice forms, even at cryogenic temperature, and the formation of ice is prevented by the presence of high concentration of chemicals that interact strongly with water and therefore, prevent water molecules from interacting to form ice (Pegg and Diaper, 1990). Vitrification of cells is a potentially less damaging procedure than freezing because it avoids the formation of intracellular ice and damaging osmotic effects that occur as a result of ice formation during equilibrium cooling and warming. Rapid cooling in high concentrations of penetrating cryoprotectants enable high rates of survival of mouse embryos of all developmental stages (Shaw *et al.*; 1991a). Vitrification protocols requires the application of a highly concentrated vitrification solution which sufficiently dehydrates cells to form a stable glass along with the surrounding vitrification solution when plunged into LN<sub>2</sub> without causing injury (Sakai *et al.*; 1990). Attempts at cryopreservation of fish embryos using vitrification has only been performed so far with zebrafish embryo as a model system (Lui *et al.*; 1998, Zhang and Rawson, 1996). The

study has shown that intermediate embryo developmental stages at 50% epiboly to prim-6 stages provided best results. There is recent report on relatively successful vitrification of Japanese flounder (*parelichthys olivaceles*) embryos in a concentrated mixture of CPAs: 20% propylene glycol with 13% methanol. Hatching rate of 5% was reported in this study (Chen & Tian, 2005). 3-hr post-thaw survival and movement of a single zebrafish embryo, microinjected with sucrose to 0.5m concentration and cooled at 0.3 °C/min to -25 °C was reported by Kopeika *et al* (2006). Vitrification approach offers a great potential for developing cryopreservation procedures for complex tissues and organs, acquiring less specialised or expensive equipment and circumventing problems of chilling sensitivity of some specimens.

### **1.2.0 Application of cryobiology**

Cryobiological techniques have application in genetic research, livestock breeding, infertility treatment, and organ transplantation. A related field, cryogenics, is devoted to the study of low temperatures effects. In the twentieth century, scientists began applying cryogenic techniques to biological systems. They explored methods for treating blood, semen, tissue, and organs with ultra-low temperatures.

The main applications of cryobiology are in the areas of biomedicine and conservation.

### 1.2.1 Application in biomedicine

Animals and cell cultures derived from animal tissues have been used for testing of vaccines and research in biomedicine. The use of cell lines for the manufacture of a range of biological medicines has brought about more reproducible and safe cell substrates (Griffiths and Doyle, 2000). Animal cell lines are very important in the establishment of *in vitro* methods for diagnosis, treatment of animal and human diseases, providing substrates for biological assays of vaccine potency and detection of adventitious agents in products. Mammalian cell lines are amenable to cryopreservation and could be stored in a stable state at ultra-low temperature using cell banks and cell banking procedures are vital preliminary step for any application of cell lines. Virus can affect the characteristics of the animal cell products used in treating patients by introducing growth media components of animal origin or from the tissue of origin of the cell culture (Erickson et al., 1991, Hallauer *et al.*, 1971, Frommer et al; 1993). Freeze- thaw cycles are used to promote release of intracellular virus and enhance virus detection.

Cryopreservation techniques for *in vitro* manipulation of human reproductive cells for the treatment of infertility have brought about a number of development in science of cryobiology. The ability to store the reproductive cells and embryos in the frozen state has greatly facilitated the progression of clinical assisted reproduction esoteric research into mainstream patient treatment. In Biomedical cryopreservation has been suggested as an ideal solution to the problem of restoring fertility in female patients who must undergo ablative chemotherapy or radiotherapy during cancer therapy



(Gosden and Aubard, 1996; Hovatta *et al.*, 1997). The treatment of human infertility has embraced IVF, a technique that allows relatively few spermatozoa to achieve fertilization. Control in interaction of spermatozoa and oocyte has resulted in many subfertile couples being enabled to conceive a child. Many who were incapable of conceiving with natural fertilization or artificial insemination (AI) are now interested in storing sperm for IVF treatment (Trombetta *et al.*; 2000). Oocyte cryopreservation may offer the oncology patient an opportunity to “bank” her eggs prior to initiation of treatment that could result in permanent ovarian damage, she may rely on these banked gametes to allow her to bear offspring (Van Uem *et al.*, 1987). A comprehensive study on fundamental research on ovarian tissue cryopreservation was conducted in 1950s in London, under direction of Sir Alan Parkes (Parkes, 1957, 1958, Parkes and Smith, 1953, 1954) on animal tissues. These early studies established many important principles concerning ovarian tissues cryopreservation, including the fact that the tissues could be damaged by exposure to the CPA alone without freezing, if expose for a long time.

Cryoprotectants are used to preserve cell culture and also enhances the survival of cell-free virus and intracellular virus. Cell banks are established to cryopreserve cells produced from initial stock of frozen cells. These cell banks are subjected to quality control routines for all cell banks which includes viability testing, testing for mycoplasma and other microbial contamination (Cord *et al.*, 1992, Stacey and Stacey, 2000). There are restrictions placed on the maximum number of population doublings permitted

between master cell bank and the cells used for the production processes in biological medicines and this is important where human diploid fibroblast cultures are used that have a finite lifespan in vitro (Wood and Minor, 1991). Patent application based on a novel cell line or involving the use of a cell line as a critical part of the patent may require that samples of the cell line or a representative cell bank must be submitted to a recognised patent depository (Fritze and Weihs, 2001). The depositories act as independent laboratories to test and hold those cultures and act as reference point for any procedure to verify or challenge the veracity of the patent. The most important stimulus for cryopreservation of spermatozoa was realization that HIV could be transmitted via semen (Stewart *et al.*, 1985). It is now mandatory that semen samples for donor insemination be cryopreserved for a minimum of 6 months while donor is tested before semen collection and 6 months later for absence of seroconversion which is the development of detectable antibodies in the blood directed against an infectious agent.

Cryosurgery is the application of freezing to destroy unwanted tissues. This technique falls under category of thermal therapy. In biomedicine it has been used in treatment of carcinomas of the breast and uterine cervix using ice saline solution (Arnatt, 1851) and routinely to treat malignancies on the surface of the body (dermatologic tumors). The freezing agent, liquid nitrogen vapour can be applied in a variety of ways, using sprays and cryoprobes. Sprays are mostly used for topical applications whilst cryoprobes are used for deep solid tumor masses. New adjuvant are being sought to increase the destructive effect of freezing, during cryosurgery of prostate and other organs

such as kidney, liver or brain, magnetic resonance imaging can be used to monitor the extent of the cryosurgical ice ball and this iceball predicts the outcome of the procedure (Hoffmann and Bischof, 2002). Cryosurgery has been shown to be potent method of *in situ* tissue destruction.

### **1.2.2 Application in conservation**

In modern reproductive technologies, there is possibility of using frozen and stored germ plasma to support conservation measures for the maintenance of genetic diversity in threatened species. There have been encouraging results following artificial insemination (AI) of frozen thawed semen at some of the zoological parks around the world. Sperm, eggs and embryos germ plasma cryopreservation has contributed directly to animal breeding programs which have helped conservation. Moreover, cryopreservation of germ plasma is a very good ex-situ strategy to conserve existing allelic diversity for future use.

Conservation of wild species was considered twenty years ago (Veprintsev & Rott, 1980) and since then many collections of genetic material have been established. Genetic resource banks (GRB) and biological resource banks are systematic ways of collecting biological material both for germplasm used in animal breeding programs and banks of somatic tissues collected for scientific research (Holt *et al*; 1996, Wildt *et al.*, 1997, Holt and Pickard; 1999).

Live offspring have been produced by the transfer of frozen or vitrified embryos in laboratory, mice (Wood *et al*; 2001) rats (Stein *et al*; 1993)

hamsters (Hane *et al.*, 1999, Ridha and Dukelow; 1985) and Mongolian garbils (Mochida *et al.*, 1999).

To achieve success in all forms of assisted reproductive technology in wild species whether or not the use of cryopreserved materials is involved, long-term storage and utilization of cryopreserved germ plasm could extend the population's generation length and allow higher levels of genetic variation to be maintained in smaller populations.

Loss of genetic variability, known as inbreeding has serious consequences for reproductive performance (Rousset, 2002, Ryan *et al*; 2003, Taylor, 2003). When populations decline, they inevitably increase the likelihood of generating inbred individuals. The ability to manage genetic diversity using cryopreserved germ plasm is an advantageous feature to conservation. It can be possible to protect adaptations that provide the ability to occupy specific ecological niches by cryopreserving material from individuals expressing the same phenotypes. Advances in reproductive technology and better understanding of the reproductive physiology of animal populations are necessary to permit routine application of artificial insemination and embryo transfer using frozen-stored germ plasm. For the last seven years, there have been a number of live-born elephant calves, both African and Asian, conserved by artificial insemination (Schmitt *et al*; 2001) and more on going pregnancies resulting from artificial insemination in the captive populations of these species. Cloning and nuclear transfer have led many technologists to suggest that endangered and extinct species like livestock and laboratory

species can be propagated and recovered, for example, the Mauritius kestrel population declined to two individuals but has shown remarkable recovery to about two hundred breeding pairs (Groombridge *et al*; 2000). Assessing the prospective viability of a threatened population is an important step in the development of conservation plans, and might indicate the need to establish protected area to help species recovery. Banking of genome resources by cryopreserved male germ plasm would greatly help conservation efforts for endangered species and captive breeding programs.

### **1.3 Current status of aquatic species cryopreservation**

Aquatic species cryopreservation has brought about the possibility of preserving specific species, increasing the representation of genetically valuable animals. Cryopreservation of reproductive materials of many aquatic species has been successful using gametes (Barros *et al.*, 2001, Billard and Zhang, 2001, Clark *et al*; 1996, Stoss; 1983, Paniagua *et al*; 2000). In the last three decades cryopreservation of fish gametes has been studied extensively and there have been successful cryopreservation of the spermatozoa from many species such as cyprinid, acipenseridae, salmonid and silurids (Maise, 1996, Rana and Gilmour, 1996, Magyary *et al.*, 1996, Lahnsteiner; 2000). Currently, studies have been carried out on cryopreservation of aquatic invertebrate semen including species from molluscs, crustacean, echinoderms, and polychaetes (Gwo, 2000).

There have been successful reports on cryopreservation of embryos of aquatic species including the embryo of pacific oyster (Lin and Chao, 2000); embryos of hard clam (Chao *et al*; 1997); juveniles of marine polychaetes (Olive and Wang, 1997) and embryos of rotifers (Toledo and Kurokura, 1990). Survival of most of these species has been reported after freezing to cryogenic temperature, but only a fraction of surviving embryos develop normally. A few embryos have been shown to survive for a short period of time after cooling to liquid nitrogen, these results could not be repeated and successful cryopreservation of fish embryos still remains elusive (Zhang *et al*; 1989, Robles *et al*; 2005, Chen and Tian, 2005, Kopeika *et al.*, 2006) Fish germplasm plays a significant role in human genomic studies because its relatively small size of the genome makes it easier for sequencing and ideal models for studying human disease. This will help in identifying roles for human genes from fish mutations and also in fish models for genes identified by human disease (Brownlie *et al.*, 1998, Barbazuk *et al.*, 2000). Cryopreservation would maintain germplasm free of genetic contamination and degradation (Rana, 1995). Aquatic species preservation will assist the development, protection and distribution of research lines and offers benefits for restoration of endangered species.

### 1.3.1 Sperm cryopreservation

A few studies have addressed sperm cryopreservation in aquarium fish. More than 200 fish species have been studied for sperm cryopreservation (Rana and Gilmour, 1996). The present state of the art for many species of fish are adequate for the purpose of gene banking. Cryopreservation of fish sperm has become routinely used to preserve genetic material and conserve genetic diversity. Cryopreserved spermatozoa of marine fish species have been more successful when compared with those obtained from fresh water fish (Tsvetkova *et al.*, 1996). Fertilization rates obtained with cryopreserved fish sperm from marine species are comparable to those obtained with mammalian species. High fertilization rates by cryopreserved sperm have been achieved in several fresh water species (Lahnsteiner *et al.*; 1995, 1997). Controlled slow cooling has been mainly used for fish spermatozoa cryopreservation. About 12 salmonid species spermatozoa have been successfully cryopreserved (Billard and Zhang, 2001; Lahnsteiner, 2000; Maisse, 1996; Tiersch and Mazik, 2000). Another well studied and successfully cryopreserved group are cyprinids and some of these cyprinid fishes are widely farmed in Asia and Europe. The application of cryopreservation techniques is important for those species with long life cycles and in the species whereby male fish only mature when they are several years old. Attempts to cryopreserve sperm of marine species have been more successful when compared with those obtained with fresh water fish (Gwo, 2000).

Cryopreservation can be used to improve existing hatchery operations by providing sperm on demand and simplifying the timing of induced spawning. Frozen sperm can enhance use of facilities and create new opportunities in the hatchery by eliminating the need to maintain live males, potentially freeing resources for use with females and can also be used in breeding programs to create improved lines and shape the genetic resources available for aquaculture (Tiersch *et al*; 2007). Sperm cryopreservation has revolutionized the field of assisted reproduction and provides hope for men undergoing chemotherapy, radiation or radical surgery who once had no chance for future fertility.



### 1.3.2 Oocytes cryopreservation

Cryopreservation of fish oocytes would be a major development enabling fish culturists to have a ready supply of embryos for use when needed. Cryopreservation of fish oocytes has been recently studied (Isyeva *et al*; 2004, Plachinta *et al*; 2004, Zhang *et al.*, 2005). Several factors may complicate the successful development of cryopreservation protocols for fish oocytes: (a) fish oocytes are large, resulting in much lower surface area to volume ratio and this reduces the rate at which water and cryoprotectant move into and out of oocytes during cryopreservation, (b) the possible susceptibility to chilling injury, (c) there can be low permeability of membrane and envelopes, and (d) the presence of yolk which may have different osmotic properties (Liu *et al*; 2001). In cryopreservation of fish oocytes quantitative estimation of oocytes viability is needed. This is required for assessing the effect of cryoprotectants, cooling to cryogenic temperature, post-thawing viability and the effect of chilling. Staining of oocytes with trypan blue, thiazol blue (MTT) and CFDA has been confirmed effective for late stage III or stage IV oocytes (Lubzen *et al*; 2003).

The permeability of the zebrafish (*Danio rerio*) oocyte membrane to water and cryoprotectants has been studied and fish oocyte membrane permeability parameters were reported for the first time by Zhang *et al* in 2005. Stage III and IV were used for the study. The volumetric changes of stage III oocytes in different concentrations of sucrose solutions were measured for 20 min exposure at 22°C and the osmotically inactive volume of the oocyte was

determined using the Boyles Van't Hoff relationship. Oocytes were exposed to different cryoprotectant solutions and volumes were measured, recorded and analysed using computer aided real time video microscopy. The study showed that the membrane permeability of stage III oocytes decreased significantly with temperature. The hydraulic conductivity ( $L_p$ ) and solute permeability ( $P_s$ ) value obtained for stage III zebrafish oocyte are generally lower than those obtained from aquatic invertebrates and higher than those obtained with immature medaka oocytes (Valdez *et al.*, 2005) or fish embryo (Zhang and Rawson, 1998). Zebrafish oocyte development is classified into five according to Selman *et al* (1993). Stage I (primary growth stage) stage II (cortical alveoli stage) stage III (vitellogenic stage) stage IV (maturation stage) and stage V (mature eggs). Each developmental stage of zebrafish oocyte has its specific characteristics of membrane composition, organelles organisation, protein content and lipid distribution. Chilling sensitivity in zebrafish oocytes was linked to lipid phase transition of the oocyte membrane (Pearl and Arav, 2000). Pearl and Arav results show phase transition in small and large zebrafish oocytes exposed to temperature between 12 and 22°C, showing that chilling damage may occur at temperatures well above the water freezing temperatures and the lipid composition of membrane (Drobnis *et al*; 1993).

Cryopreservation of fish oocytes has several advantages over fish embryos, such as the small size of the oocytes, the relatively low water content in oocytes of pelagic spawners and the absence of a fully formed chorion that may render the oocyte more permeable to solutes than the egg. Fish oocytes are relatively smaller than their respective mature eggs and constitute a single compartment and do not lower permeability barriers, such yolk syncytial layer

are found in embryos (Hagedon *et al.*, 1996, 1998). The yolk content was shown to be implicated in chilling sensitivity of zebrafish embryos (Liu *et al.*, 1999) and perhaps contributes to the chilling sensitivity of zebrafish oocytes (Isayeva *et al.*, 2003).

### 1.3.3 Embryo cryopreservation

Cryopreservation of embryos has become an integral part of assisted reproduction. Successful cryopreservation of embryos is important because the biodiversity of both paternal and maternal genomes will be preserved. Over 10 species have been used in conducting fish egg and embryo cryopreservation for the past 20 years. Some of the species used are rainbow trout (*Salvelinus Fontanelis*; Zell, 1978) meaka (*Oryzias Latipes*; Onizuka *et al*; 1984). African catfish (*Clarias gerrie pinus*; Magyary *et al*; 1996). Zebrafish (*Danio rerio*, Harvery, 1983; Zhang & Rawson, 1996a; Zhang *et al*; 1993), herring (*Chipea harengus*, Ben-Amotz & Rosenthal, 1981&Whittingham and Rosenthal, 1978), turbot (*Scophthalmus maximus*; Cabrita, *et al*; 1999).

Some factors have limited fish embryos cryopreservation and they are the compartmental structure, high chilling sensitivity and low membrane permeability and their large size which gives low surface area to volume ratio. The fertilised eggs of most fish species are greater than 1mm in diameter. The effect of such low ratio is a reduction in the rate at which water and cryoprotectants can move into and out of the embryo during cryopreservation (Mazur, 1984). Fish embryos are osmoregulators, they are released into the

external medium and activated, the vitelline envelope separates from the plasma membrane and forms chorion. The studies of chorion (Hart and Donovan, 1983; Kalicharan *et al*; 1998; Zhang *et al*; 1993) of zebrafish embryos showed the chorion to be a thin envelope constructed of three zones; an inner zone of lower electron density rich in protein, an outer, electron-dense zone containing pore canals rich in polysaccharides (Tesoriero, 1977) and a middle fibillar zone. Studies on the chorion permeability (Hisaka, 1955; Zhang and Rawson, 1996b) of zebrafish eggs clearly showed it is permeable to electrolytes and a range of cryoprotectant such as propane-1,2-diol, methanol, DMSO, ethylene glycol. The chorion structure plays a role as flexible filter for transport of some materials (Toshimori and Yasuzumi, 1976) and protects against micro organisms (Schoots *et al*;1982). It may also play a role in diffusive exchange of gases as well as providing physical protection (Grierson & Neville, 1981). The chorion is freely permeable to water and small molecules but the permeability of the plasma membrane gradually increases again but it remains significantly lower than before activation and fertilization. When fish eggs are released into the external medium and activated by the sperm or by the change in external medium composition, the vitelline envelope separates from the plasma membrane and forms the chorion. Between these two membranes the perivitelline space forms, made up of collids and enzymes released from the cortical alveoli and water imbibed from the medium (Coward *et al*; 2003). The chorion is a freely permeable to water and small molecules but the permeability of the plasma membrane decreases markedly following activation and fertilization (Rawson *et al*; 2000). Two cryopreservation methods used so

far for fish embryo are controlled slow cooling and vitrification, although none of the two has yet been successfully employed to cryopreserve fish embryos. Slow cooling rates continuously result in higher survival for fish embryos as compared to high cooling rate. The optimum cooling rates reported were in the range of 0.01 to 0.75°C/min (Onizuka *et al*; 1984; Stoss and Donaldson, 1983; Harvey and Ashwood-Smith, 1982; Zhang *et al*; 1993). Cryopreservation studies have shown that intermediate embryo development stages between postgastrula and heartbeat have higher survival rate. Although a few embryos have been shown to survive for a short period of time after cooling to liquid nitrogen, these results can not be repeated and successful cryopreservation of fish embryos remains elusive (Zhang *et al*., 1989, Robles *et al*; 2005, Chen & Tian, 2005).

#### **1.4 Use of zebrafish (*Danio rerio*) as a model**

World- wide fish populations are threatened by over- fishing and environmental pollution. In Europe more than 65% of fish species are threatened (Kirchofer, 1996). Fish germplasm plays significant role in human genomic studies. Efficient sperm cryopreservation enhances the zebrafish model system by optimizing productive use of facility space, extending the reproductive life time of male, providing an alternative to live stocks for strain recovery, and ensuring the survival of culture of zebrafish (*Danio rerio*) has made it a favourite model system for biologist studying embryonic development. Large numbers of mutations that disrupt embryonic

development have now been isolated in the zebrafish, many of which may serve as models for human disease syndromes. The relatively small size of fish genome makes them easier for sequencing and ideal models for studies on gene evolution and human disease. Understanding the relationship between fish and human genomes will help identify roles for human genes from fish mutations and help identify fish models for gene identified by human disease (Barbazuk *et al.*, 2000, Brownlie *et al.*, 1998). Zebrafish have a unique combination of genetic and experimental embryologic advantages that make them ideal for studying the embryogenesis of the circulatory system.

There are numerous advantages of using zebrafish as a model namely:

Fish plays a significant role in human genomic studies because they contain essentially the same genes as human. Adult zebrafish are small (approx. one inch long as adults). They have short generation time (3-4 months). They have high fecundity (mature female lay hundreds of eggs at weekly intervals). They easily bred in large numbers and have rapid development. It requires as few as 50 cells for transplant. It undergoes external fertilization. It is inexpensive and easy to maintain. Embryos are translucent. It permits *in vivo* visualization of cell migration. They require small amount of drug per experiment  $\mu\text{M}$  vs  $\text{mM}$ . Screening zebrafish population has identified mutations in a number of genes- including sonic hedge hog- that result in a phenotype reminiscent of the holoprosencephaly syndrome. These mutants allow the entire genetic network responsible for this conditions to be examined in a developing system that closely resembles mammalian development. The zebrafish genome is about  $1.7 \times 10^9$  base-pair, compared

with mammalian genome size of about  $3.2 \times 10^9$  base- pair. Majority of these studies have been carried out with gametes (Barros *et al.*, 1997, Billard and Zhang, 2001&Stoss,1983, Clark *et al.*, 1996, Paniagua *et al.*, 1998).

### **1.5 Aim of present study**

Cryopreservation of fish oocytes has not been studied systematically and cryopreservation studies have only been carried with stage III oocytes. In this present study cryopreservation of I and II oocytes will be studied using controlled slow cooling. Toxicity of cryoprotectants to stage I and II zebrafish oocytes, will be investigated, the optimal cooling rate for these oocytes and the optimal conditions for removing cryoprotectants, will be identified in developing a optimum cryopreservation protocol for zebrafish oocytes at early stages.

## 2.0 MATERIALS AND METHODS

### 2.1 Introduction

The aim of the study was to investigate the toxicity of cryoprotectants to stage I and II zebrafish (*Danio rerio*) oocytes, identifying the optimal cooling rate for these oocytes and establish the optimal conditions for removing cryoprotectants. Oocytes were treated using different cryoprotectants in the toxicity studies: methanol, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG). Solutions of cryopreservative agents (CPAs) were made up in KCl-buffer (55mM KCl; 55mM K acetate; 1mM MgCl<sub>2</sub>; 2mM CaCl<sub>2</sub>; 10mM HEPES; pH 7.4) at a range of concentrations: 1M, 2M, 3M, 4M and 5M. The experiments were carried out in the laboratories at the Institute, of Research in the Applied Natural Sciences (LIRANS) University of Bedfordshire.



## **2.2 Methods for zebrafish care**

### **2.2.1 Maintenance of zebrafish**

Zebrafish (*Danio rerio*) were obtained from Aquascope Ltd. Birmingham. Fish were maintained in filtered aerated 40L tanks at 28°C with a light/dark cycle of 12/12. Tap water, which was aged for 2 days to release chlorine was used and aerated constantly. Aeration of water in the tank was carried out by using an electric pump to pump air under an upturned funnel that was surrounded by filter floss in a beaker immersed in the fish tank. Water was pulled by the suction effect generated by the rising air bubbles through the smooth gravel.

### **2.2.2 Feeding**

The fish were fed twice a day with 'Tetramin' (Tetra, Germany) dry fish food, a supplement of live adult brine shrimp was also provided in the middle of the day.

## **2.3 Collection of oocytes**

The present study was carried out using stage I and II zebrafish oocytes, stage III oocytes were also used as comparison.

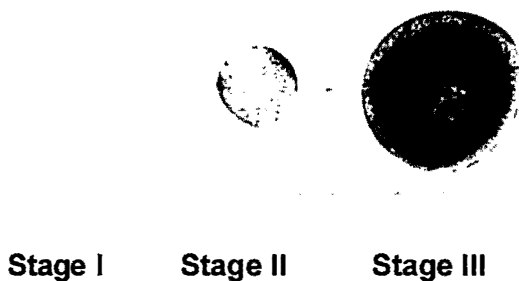


**Fig. 2.1. Female adult zebrafish (*Danio rerio*)**

The gravid female zebrafish (Fig. 2.1) were collected from the fish tanks.

In order to obtain stage I (primary growth stage) oocytes, stage II (cortical alveoli stage) oocytes and stage III (vitellogenic stage) oocytes zebrafish (Fig. 2.2) was anaesthetised with tricaine (0.6mg/ml) for 5min, they were then decapitated. The ovaries were removed and immersed into 1.6mg/ml hyaluronidase made up in KCl-buffer (55mM KCl; 55mM K acetate; 1mM MgCl<sub>2</sub>; 2mM CaCl<sub>2</sub>; 10mM HEPES; pH 7.4) at 22°C for 10min. Ovaries were then gently separated by using forceps and repeated pipetting to remove the interstitial cells until the ovarian cumulus were separated to single oocytes. After enzyme treatment, oocytes were washed three times in KCl-buffer and randomly distributed in wells of 6-well plates. All experiments were carried out at room temperature.

Changes in the surface area to volume ratio have important implications for limits or constraints oocytes size, and help explain some of the modifications seen in larger-bodied oocytes. Therefore the surface area to volume ratios was calculated and were 1:3, 1:4 and 1:9 for stage I, II and III respectively.



**Fig. 2.2 Zebrafish oocytes at stage I, II and III.**

Stage I (Primary growth stage) zebrafish oocytes do not exceed 140µm in diameter. They increase gradually in size to become more cubical and then adjacent cells separate from each other to form intercellular spaces. Later, two cell layers, the thecal and granulosa cells can be easily distinguished in a follicle and support further growth. The cells within the follicle are in intimate contact during the growth period.

Stage II (Early cortical alveolar stage) oocytes, the diameter ranges from 140 to 340µm. The content of cortical alveoli will be released into perivitelline space after fertilization to form perivitelline fluid and prevent multiple sperm entry. In a later phase, the ooplasm gradually loses its basophil, while a space appears between the granulosa layer and the oocytes surface (Matova & Cooley, 2001).

Stage III (Vitellogenesis) oocytes, the diameter ranges from 340 to 690µm. They can be visually observed, they are opaque and slightly yellowish in colour. At the beginning of vitellogenesis stage the zona radiata surrounding oocyte becomes more distinct. During vitellogenesis the ooplasm continues to lose its basophilicity (Wallace & Selma, 1981). Vitellogenesis regulation

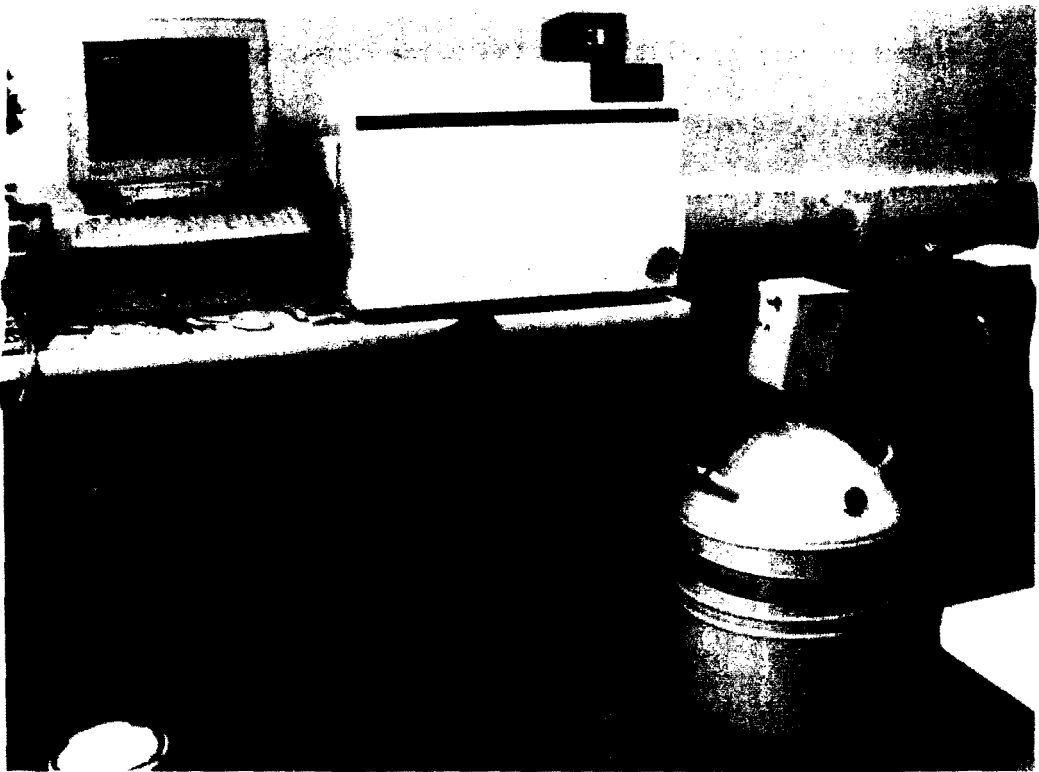
involves the interaction of the anterior pituitary in the brain, the follicle cells, the liver and the eggs.

## **2.4 Toxicity studies of cryoprotectants**

Investigations on toxicity of cryoprotectants to zebrafish oocytes have been carried out by Plachinta *et al* (2004) using stage III and IV zebrafish oocytes. The present study was carried out using stage I, II and III zebrafish oocytes to determine zebrafish oocytes sensitivity to cryoprotectant toxicity. Methanol, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol were used and solution of cryoprotective agents (CPAs) were made up in KCl-buffer at range of concentrations: METH 4M and 5M; PG 2M, 3M and 4M; DMSO 3M, 4M and 5M; EG 1M, 2M, 3M and 4M in order to identify the least toxic cryoprotectant to zebrafish oocytes. Stage I, II and III oocytes were put into each well of the 6-well culture plates containing CPAs and were incubated for different time periods: 30, 60, 120 and 180min at 22°C. Control oocytes were incubated in KCl-buffer solution under the same conditions. After incubation in CPA solutions, oocytes were washed twice with KCl-buffer and viability tests were conducted. 20-50 oocytes were used in each replica, 3 replicas were used in each experiment and the experiments were repeated at least 3 times.

## 2.5 Cryopreservation of zebrafish (*Danio rerio*) oocytes using controlled slow cooling

Investigations on cryopreservation of zebrafish oocytes using controlled slow cooling have been carried out by Planchinta *et al* (2004b, 2005) using stage III (vitellogenic) oocytes. The present study were carried out using stage I and II zebrafish oocytes to identify the optimal cooling rate and optimal conditions for removing cryoprotectant. Methanol was used in these experiments because it was the least toxic cryoprotectant to zebrafish oocytes identified from the toxicity studies and previous studies by planchinta *et al*; 2004.



**Fig. 2.3 Programmable cooler (Planner KRYO 500) used in controlled slow cooling.**

In these experiments, 20-50 oocytes were put into each well of the 6 well culture plates. Stage I and II oocytes were exposed to 4M methanol made up in KCl-buffer for 30min at 22°C. After 30min incubation, oocytes were then loaded into 0.5ml plastic straws in 3 replicas and put into a programmable cooler (Fig. 2.3) (Planner KRYO 550). Oocytes were cooled using the following protocol (1): Starting temperature was set at 20.0°C and subsequently cooled to -12.5°C at cooling rate of 2.0°C/min, (2): seeding at -12.5°C and samples were held at this temperature for 10min (3): samples were then cooled at 0.3°C, 0.5°C or 1°C/min respectively, to temperature of -40.0°C, (4): subsequently 10°C/min to -80.0°C followed by plunged into liquid nitrogen (LN) and held for at least 10min. After freezing, the straw containing the oocytes were thawed from -196°C to 20°C using a water bath at 27°C (>300°C/min) for 10 sec. Cryoprotectants were removed in 1 step before they were finally transferred to KCl-buffer medium for 10min at room temperature and viability test were conducted using trypan blue (TB) staining at room temperature. The experiment was repeated at least 3 times.

Further investigations were carried out using the same cooling protocols. Oocytes were cooled using 1°C/min cooling rate, after seeding to -40°C. They were then kept in KCl-buffer solution after thawing for a longer period of time, 60 and 120min and viability test were conducted using TB staining at room temperature. These experiments were also repeated at least 3 times.

## **2.6 Oocytes viability assessment**

Trypan blue (TB) staining was used to assess oocytes membrane integrity. Stage I and II oocytes viability were assessed using TB staining. After cryoprotectant treatments at 22°C and freezing in methanol to -196°C, oocytes were stained in 0.2% trypan blue solution at room temperature (22°C) for 5min and then washed with KCl-buffer. Total oocyte and viable oocyte counts were carried out under the microscope. Stained cells were considered non-viable and unstained viable.

## **2.7 Data handling**

### **2.7.1 Statistical analysis**

For all experiments, three replicas were used for each experiment. Where replicas are quoted numerically and the standard error is indicated by the '±' values. In graphs the standard error is shown by a bar. One way ANOVA followed by scheffe's post-hoc tests was used for statistical analysis ( $p < 0.05$ ). Data presented are means and standard errors.

## 3.0 Results

### 3.1 Introduction

The results reported here are on the investigations carried out to identify the optimal cryoprotectant (type and concentration), the appropriate rate of cooling to cryogenic temperature and optimal conditions for removing cryoprotectants in fish oocytes. Cryoprotectants (CPAs) are substances characterised by their ability to reduce cryoinjury of biological methods during the course of freezing. It can also be toxic for cells. Optimum cryoprotectant should have low toxicity and be able to permeate the oocyte plasma membrane. Four penetrating CPAs, namely Methanol, Dimethyl Sulfoxide (DMSO), propylene glycol (PG) and ethylene glycol (EG) were chosen for the study. Methanol has been shown to be the most effective CPA for zebrafish embryo (Zhang *et al.*, 1993) and sperm for many fish species (Lahnsteiner *et al.*; 1997). DMSO is the most widely used CPA and has been used in the cryopreservation of a wide range of cell types, cells, tissues and organs. EG and PG are similar compounds but differ only by the presence of the methyl group in PG molecule. EG and PG have been widely used for vitrification of mammalian oocytes and ovarian tissue and also for controlled slow cooling programmes (Picton *et al.*, 2002). The selection of cryoprotectant used in controlled freezing experiments was based on their toxicity to oocytes. This was carried out before cryopreservation of zebrafish oocytes (stage I and II) using controlled slow cooling.



### 3.2 Toxicity studies on cryoprotectants

The No Observed Effect of Concentrations (NOEC) were determined based on statistical results of ANOVA and Scheffe's test. The NOEC means the highest concentration of a compound used in a test, that has no effect statistically on the exposed sample when compared with controls. The NOECs for methanol, dimethyl sulfoxide (DMSO), propylene glycol (PG) and ethylene glycol (EG) are >5M, >4M, 4M and 1M respectively (Table 3.1). The results showed after stage I, II and III oocytes being treated in CPAs for 30min at room temperature, the toxic effect of cryoprotectants on oocytes generally increased with increasing concentration and exposure time (Fig. 3.1-3.12). TB staining showed that cryoprotectant toxicity to oocytes at stage I, II and III increased in the order of methanol, DMSO, PG and EG. The No Observed Effect Concentrations (NOECs) for methanol, DMSO, PG and EG for oocytes at three different developmental stages using TB-assessment method are given in Table 3.1.

Table 3.1. The No Observed Effect Concentration (NOEC) of cryoprotectant of three developmental stages of oocytes obtained from TB assessment

CPA	STAGE I	STAGE II	STAGE III
METHANOL	>5	>5	>5
DMSO	>4	>4	>4
PG	4	4	4
EG	1	1	1

Oocytes were exposed for 30min to CPAs in KC&buffer at 22&C. The highest concentration of CPAs used in the experiments with stage I, II and III oocytes was 5M for methanol and PG, 4M for DMSO and EG.

3.2.1 Effect of concentration and exposure time

The comparisons of oocyte survivals using different concentrations of CPA were shown in Fig 3.1 – 3.12. Stage I, II and III oocytes were used in the experiments and Figs. 3.1 - 3.12, showed oocytes survivals after treatment in different concentrations of CPA. Stage I, II and III oocytes viability after treatment for 30min at room temperature assessed with trypan blue (TB) using different concentrations of CPA are given in the table below.

Table 3.2. Effect of different concentration of CPAs on stage I, II and III when treated for 30min at room temperature and compared with their controls at room temperature at 22°C.

		Stage I	Stage II	Stage III
Methanol	Controls	95.4 ± 1.7%	90.2 ± 2.3%	73.7 ± 1.3%
	4M	94 ± 1.9%	88.3 ± 2.5%	73.7 ± 2%
	5M	92.6 ± 2.2%	86.2 ± 2.5%	71.5 ± 2%
DMSO	Controls	88.9 ± 1.5%	79.4 ± 1.7%	75.6 ± 1.95
	3M	87.5 ± 1.9%	77.5 ± 2.8%	67.4 ± 2.5%
	4M	87 ± 1.9%	75.3 ± 2.5%	68.2 ± 2.3%
PG	Controls	87.2 ± 2.7%	81.3 ± 1.4%	60.4 ± 1.2%
	2M	86.6 ± 2.4%	80.4 ± 2.4%	62.8 ± 2.9%
	3M	87.6 ± 2%	81.2 ± 2.4%	60.8 ± 2.2%
	4M	85.1 ± 2.3%	79.6 ± 2.3%	63.5 ± 1.5%
	5M	55 ± 2.1%	63.6 ± 1.7%	53.8 ± 2.3%
EG	Controls	90.6 ± 2.5%	84.3 ± 2.7%	72.9 ± 2.1%
	1M	86.2 ± 3.4%	81.9 ± 2.6%	67.9 ± 2.3%
	2M	69.1 ± 3%	61.1 ± 3%	57.7 ± 1.5%
	3M	58.7 ± 1.9%	55.3 ± 2%	53.8 ± 1.4%
	4M	57.9 ± 2.2%	54.4 ± 2.3%	53.1 ± 1.5%

There were no significant differences in oocyte survivals when compared with their 30min controls. However, significant difference was found in oocytes survivals when oocytes were treated in 5M PG when compared to their controls. After 5M PG treatment, the survivals reduced. Oocytes treated in 2, 3 and 4M EG appeared to have very different ( $p < 0.05$ ) survival rates when compared with their controls, oocyte survivals were reduced after 30min treatment and exposure at room temperature in 2, 3 and 4M EG. The toxic effect of CPAs on oocytes generally increased with exposure time.

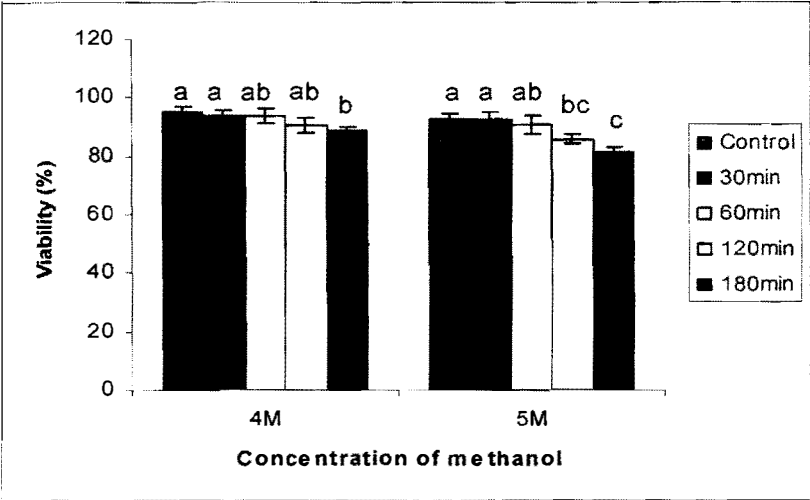


Figure 3.8. Comparisons of toxic effect of concentration and exposure time on stage I zebrafish oocytes assessed with TB staining. Oocytes were exposed to 4M and 5M methanol at different time periods.

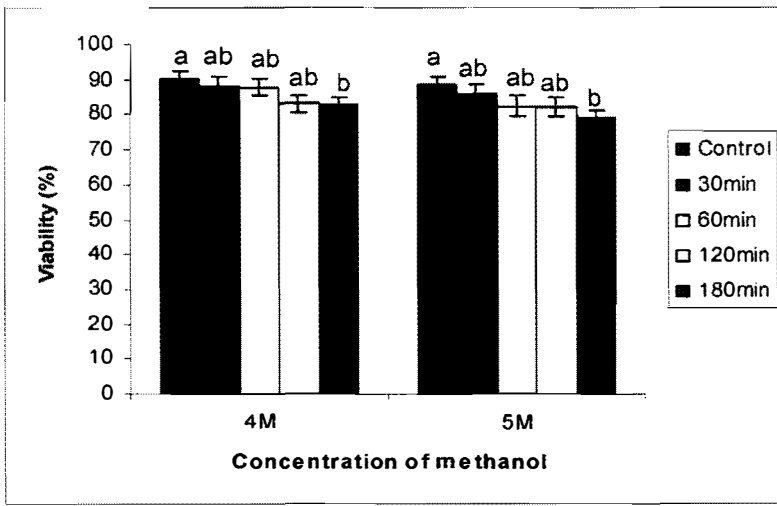


Figure 3.2. Comparisons of toxic effect of concentration and exposure time on stage II zebrafish oocytes assessed with TB staining. Oocytes were exposed to 4M and 5M methanol at different time periods.

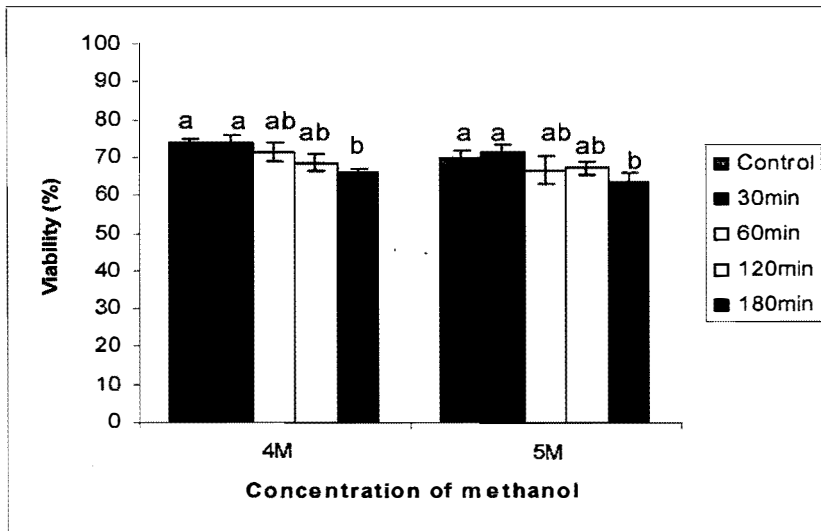


Figure 3.3. Comparisons of toxic effect of concentration and exposure time on stage III zebrafish oocytes assessed with TB staining. Oocytes were exposed to 4M and 5M methanol at different time periods.

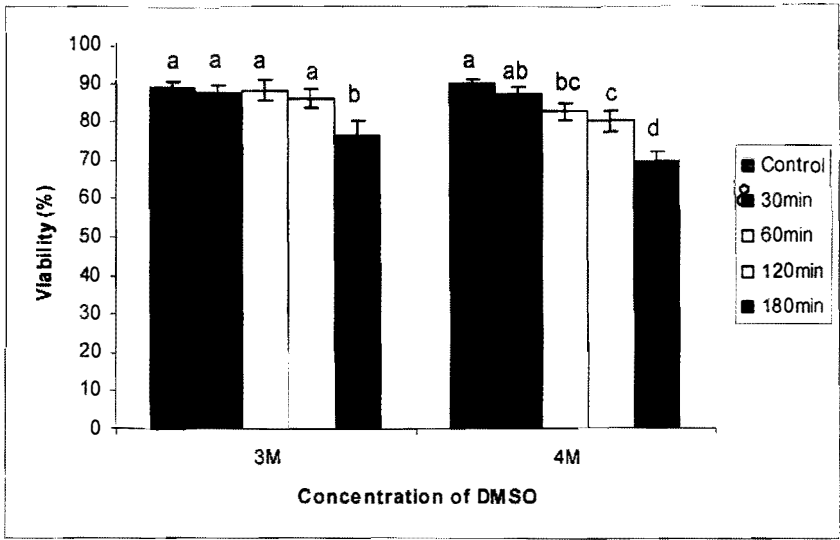


Figure 3. 4. Comparisons of toxic effect of concentration and exposure time on stage I zebrafish oocytes assessed with TB staining. Oocytes were exposed to 3M and 4M DMSO at different time periods.

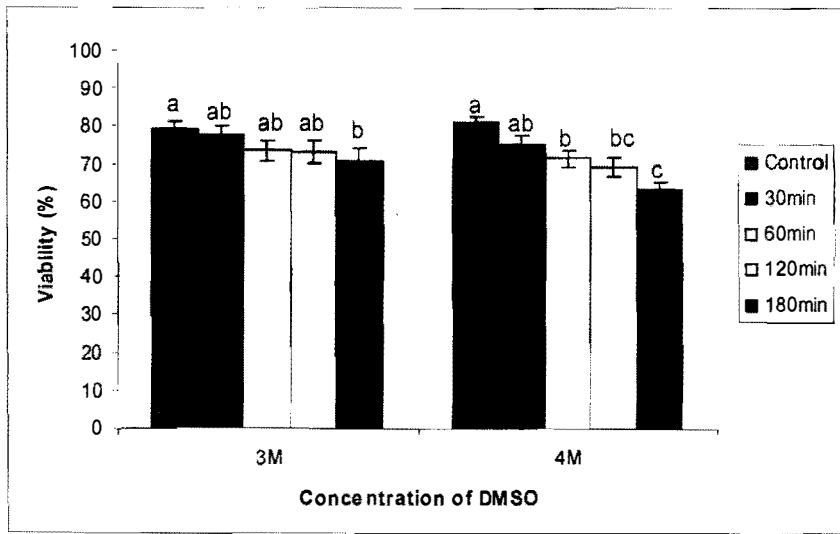


Figure 3.5. Comparisons of toxic effect of concentration and exposure time on stage II zebrafish oocytes assessed with TB staining . Oocytes were exposed to 3M and 4M DMSO at different time periods.

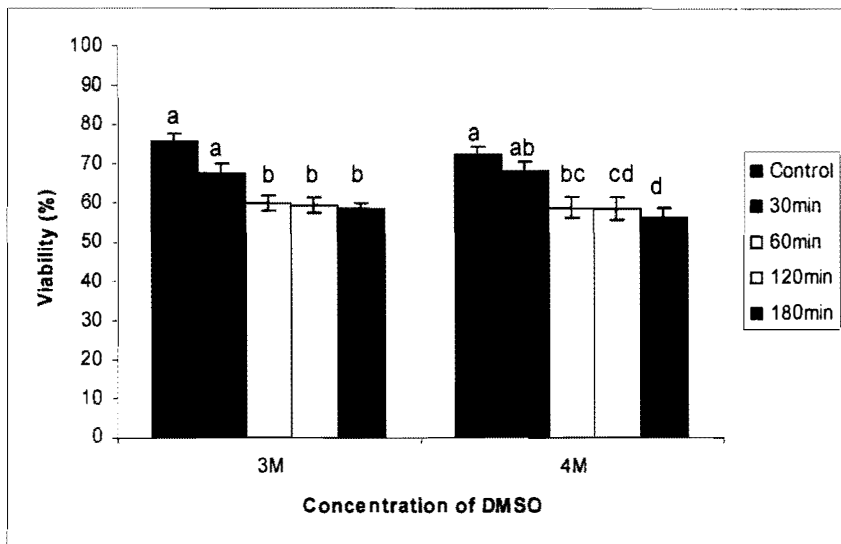


Figure 3.6. Comparisons of toxic effect of concentration and exposure time on stage III zebrafish oocytes assessed with TB staining. Oocytes were exposed to 3M and 4M DMSO at different time periods.

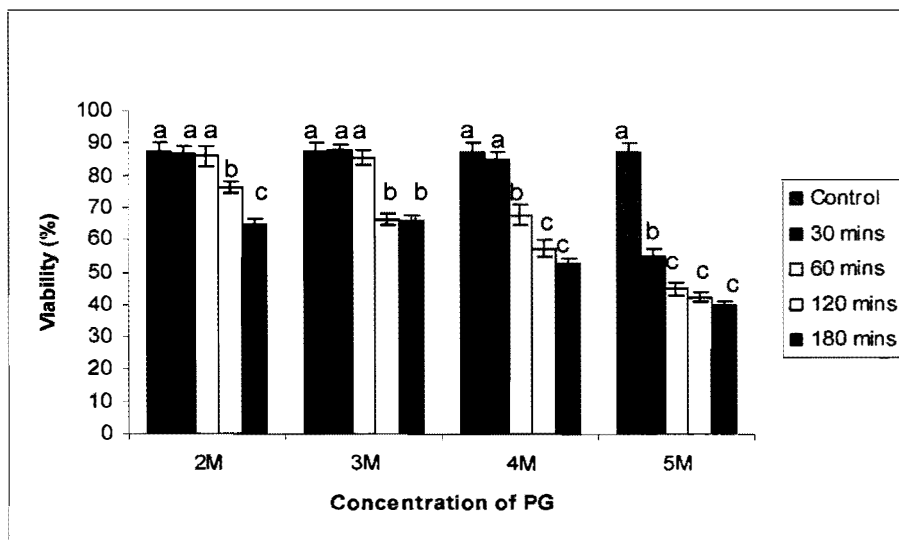


Figure 3.7. Comparisons of toxic effect of concentration and exposure time on stage I zebrafish oocytes assessed with TB staining. Oocytes were exposed to 2M, 3M 4M and 5M PG at different time periods.

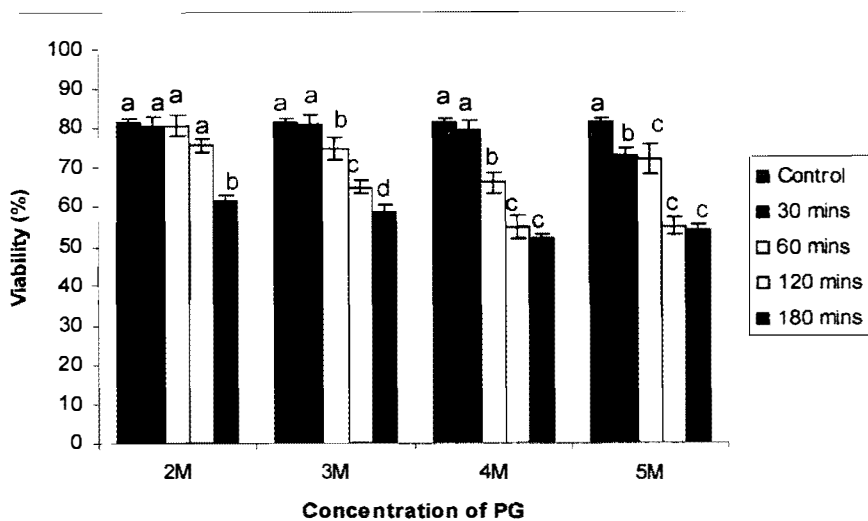


Figure 3.8. Comparisons of toxic effect of concentration and exposure time on stage II zebrafish oocytes assessed with TB staining. Oocytes were exposed to 2M, 3M 4M and 5M PG at different time periods.

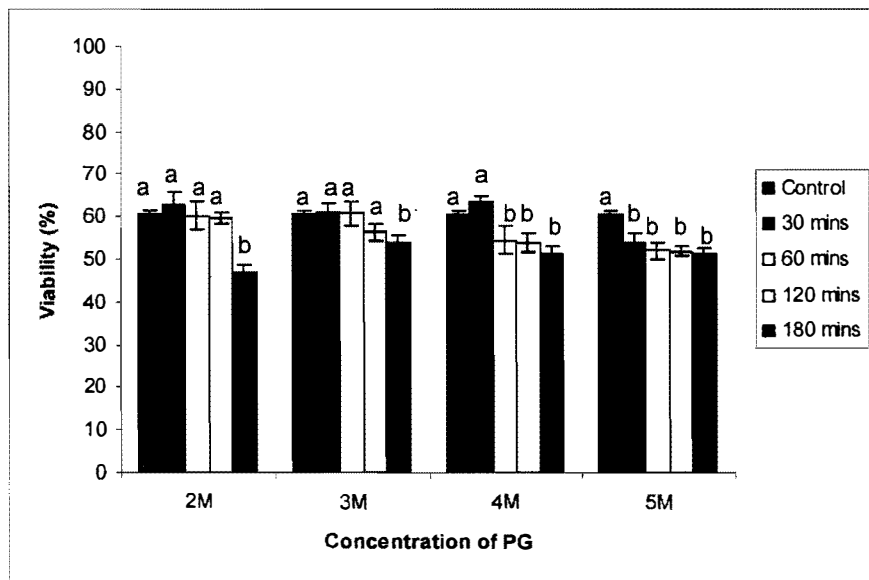


Figure 3.9. Comparisons of toxic effect of concentration and exposure time on stage III zebrafish oocytes assessed with TB staining. Oocytes were exposed to 2M, 3M 4M and 5M PG at different time periods.



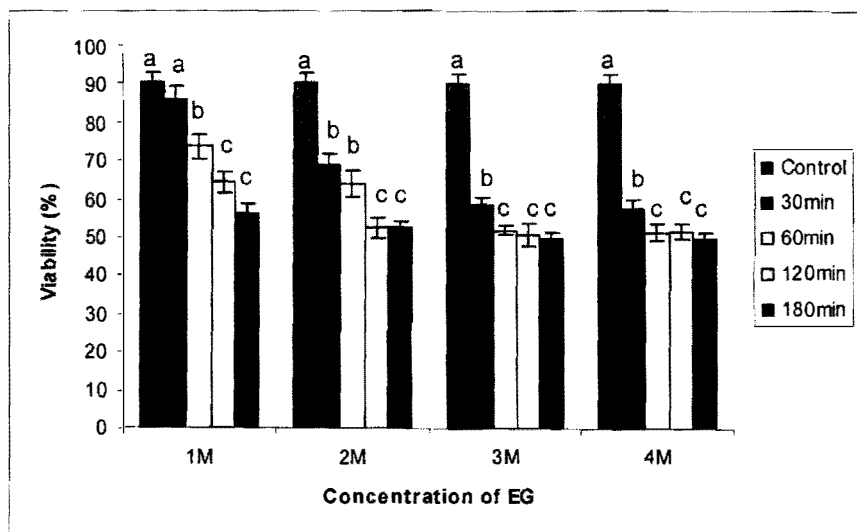


Figure 3.10. Comparisons of toxic effect of concentration and exposure time on stage I zebrafish oocytes assessed with TB staining. Oocytes were exposed to 1M, 2M, 3M and 4M EG at different time periods.

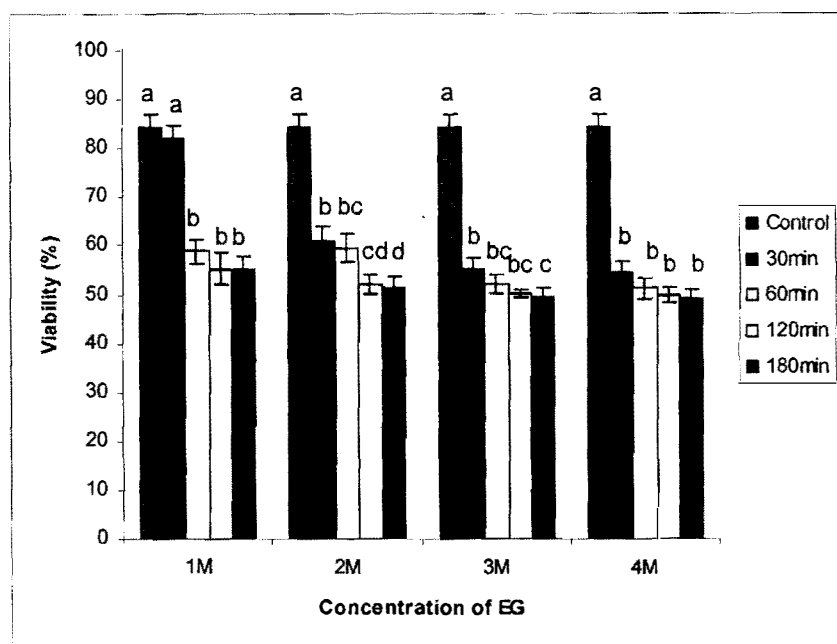


Figure 3.11&Comparisons of toxic effect of concentration and exposure time on stage II zebrafish oocytes assessed with TB staining . Oocytes were exposed to 1M, 2M, 3M and 4M EG at different time periods.

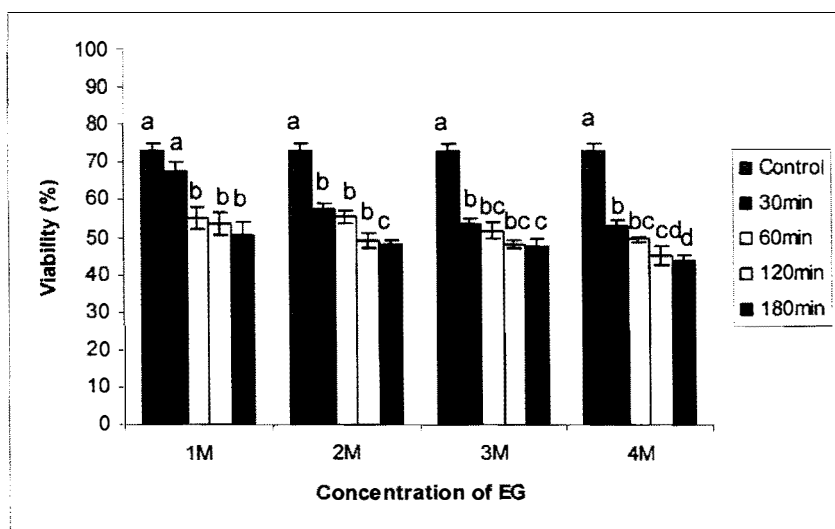


Figure 3.12. Comparisons of toxic effect of concentration and exposure time on stage III zebrafish oocytes assessed with TB staining. Oocytes were exposed to 1M, 2M, 3M and 4M EG at different time periods.

### 3.2.2 Effect of oocyte developmental stage

Stage I, II and III oocytes were used in the experiment. Experimental results showed that the sensitivity of oocytes to methanol (Fig. 3.13), DMSO (Fig. 3.14), PG (Fig. 3.15) and EG (Fig. 3.16) increased with developmental stage. The No Observed Effect concentration (NOEC) for stage I, II and III oocytes after incubation in Methanol for 30min at room temperature was >5M for the three stages (Table 3.1). The NOECs for DMSO was >4M for the three stages (Table 3.1). The NOEC for PG remained at 4M and EG at 1M for the three stages (Table 3.1). However EG is the most toxic cryoprotectant when compared to methanol, DMSO and PG. Methanol was the least toxic

cryoprotectant. The study showed that the toxic effect of cryoprotectant on zebrafish oocytes generally increased with increasing concentration (Figs. 3.1 – 3.16). There are advantages in using immature oocyte for cryopreservation rather than mature eggs. The basic cellular structures in stage I and II oocytes such as enlargement of the nucleus and appearance of multiple nucleoli and subcellular organelles plays an important role in oocyte fate. These stages include the pre-follicle phase and follicle phase. A sheath of follicle cells surrounds individual oocytes and support further growth. Chorion and vitelline envelope appears in the space between follicle layer and oocyte surface which is made up of protein and polysaccharides, synthesized both in oocytes and liver. Previous studies stated that mature fish eggs are less permeable to solutes due to loss of membrane channel protein while immature oocytes of aquatic species such as amphibian and teleost fishes are much more permeable to water and solutes due to their intact membrane protein (Ecker & Smith, 1971), and they are less hydrated than mature eggs (Wallace & Selman, 1981).

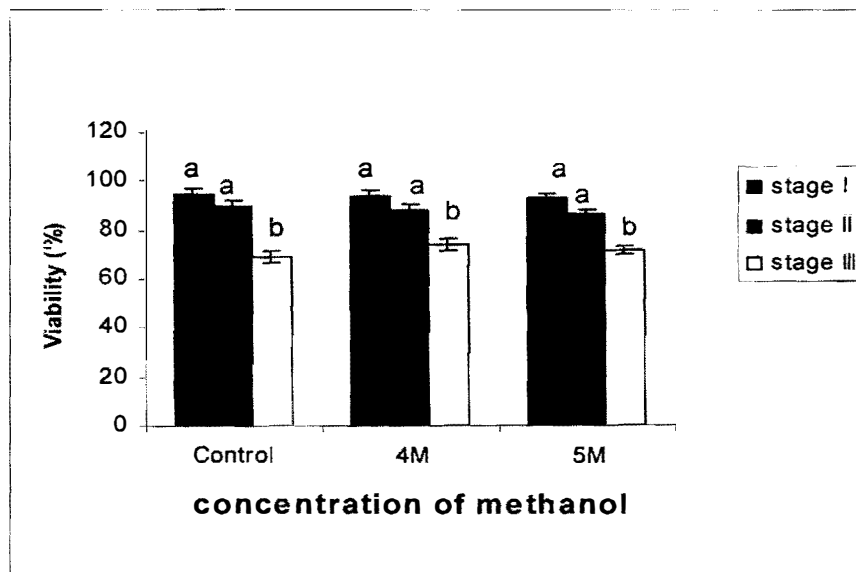


Figure 3.13. Effect of methanol toxicity on viability of oocytes at different stages, assessed with TB staining. Oocytes were exposed to methanol for 30min at room temperature.

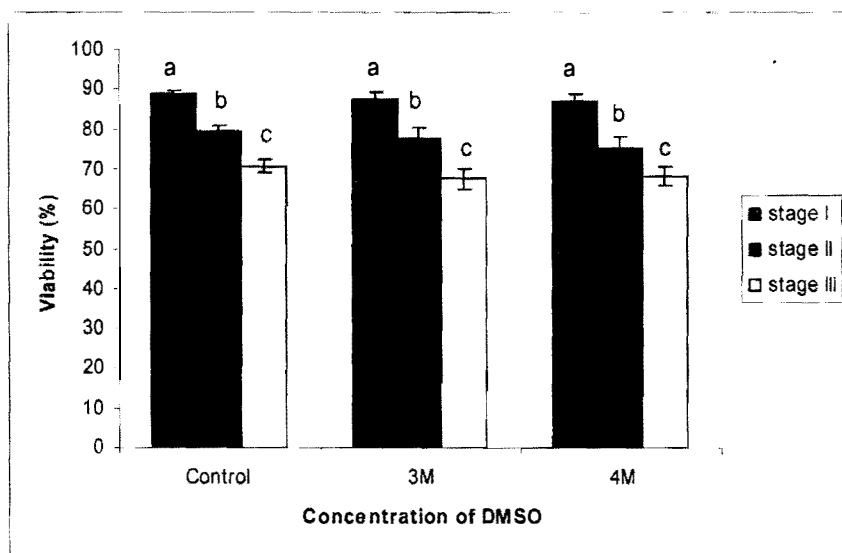


Figure 3.14. Effect of DMSO toxicity on viability of oocytes at different stages, assessed with TB staining. Oocytes were exposed to DMSO for 30min at room temperature.

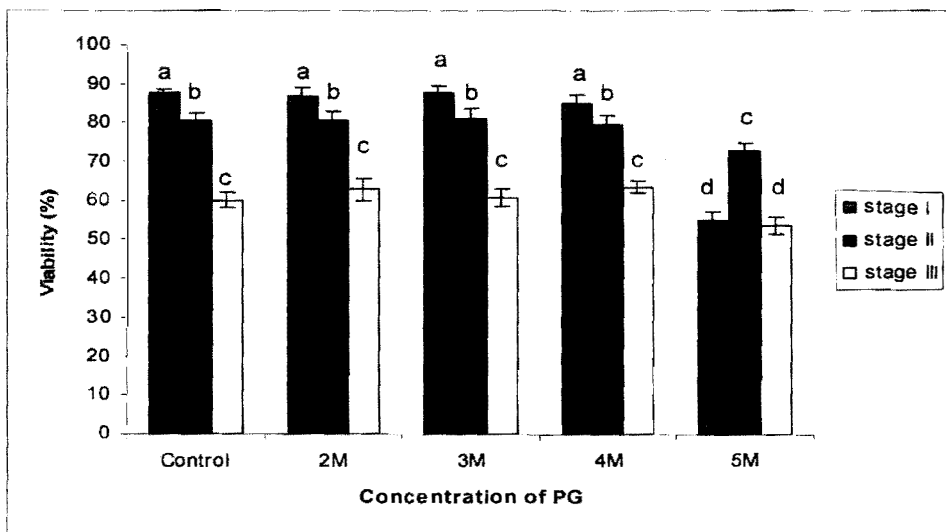


Figure 3.15. Effect of PG toxicity on viability of oocytes at different stages, assessed with TB staining. Oocytes were exposed to PG for 30min at room temperature.

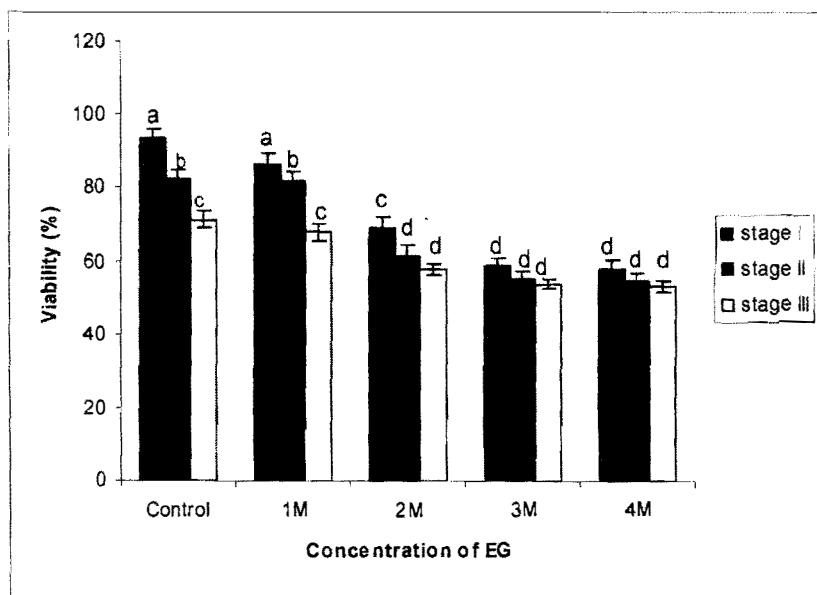


Figure 3.16. Effect of EG toxicity to viability of oocytes at different stages, assessed with TB staining. Oocytes were exposed to EG for 30min at room temperature.

### 3.3 Cryopreservation of zebrafish oocytes using controlled slow cooling

Methanol was used in the freezing experiments for zebrafish oocytes because it was the least toxic cryoprotectant following previous findings (Planchinta *et al.*, 2004) and the present toxicity studies. Controlled slow cooling was used for the cryopreservation of the fish oocytes. Slow cooling rate enables sufficient cell dehydration and decreases the possibility of formation of intracellular ice, however, if cooling rates are too slow, cells will be over exposed to solution effects and toxicity of cryoprotectants (CPAs) (Mazur *et al.*, 1984; Karlsson *et al.*, 1996). Stage I and II oocytes were used in this study as they are more permeable to water and solutes (Pennequin *et al.*; 1975., Ecker & Smith, 1971, Seki *et al.*; 2005, Smith & Ecker, 1969). Stage III oocytes were not used in these studies as they were studied before by Plachinta *et al.* (2004, 2005). In this study three slow cooling rates (0.3, 0.5 and 1°C/min) and 4M methanol in KCl-buffer were used for the experiments. Trypan blue staining was used for the assessment of their viability. In controlled slow cooling, the parameter that determined the success of cryopreservation are the cooling rate, ice-seeding temperature, liquid nitrogen plunging temperature and thawing rate.

3.3.1 Effect of cooling rate

Zebrafish oocytes were incubated in 4M methanol solution for 30min at room temperature, and were then loaded into 0.5ml plastic straws before freezing to -40°C, using slow cooling rates (0.3, 0.5 and 1°C/min).

Table 3.3. Effect of different cooling rates on stage I oocytes in 4M methanol KCl-buffer

Stage I		
Cooling rate	Control	Treatment (Survival)
0.3°C/min	96.8 ± 2.8%	60.7 ± 7.5%
0.5°C/min	98.8 ± 1.9%	69.3 ± 6%
1°C/min	96.9 ± 3.2%	87.8 ± 4.9%

The effect of oocyte survivals after freeze-thawing was investigated by using one way ANOVA test. It was shown that there were significant differences in stage I oocytes survivals observed in experiments with TB, between groups cooled at the rate of 0.3°C/min, 0.5°C/min or 1°C/min ( $p<0.05$ ) after freeze-thawing (Fig 3.17).

Table 3.4. Effect of different cooling rates on stage II oocytes in 4M methanol KCl-buffer

Stage II		
Cooling rate	Control	Treatment(Survival)
0.3°C/min	96.5 ± 2.6%	69.5 ± 4.7%
0.5°C/min	95.3 ± 4.9%	84.9 ± 5.8%
1°C/min	94.4 ± 3.8%	86.8 ± 6.5%

In stage II, it was shown that there were no significant differences between oocytes cooled at the rate of 0.5°C/min or 1°C/min when assessed with TB after freeze-thawing. Significant differences were observed for oocytes cooled at 0.3°C/min cooling rate. Oocyte survivals were significantly lower when compared with those cooled at 0.5°C/min or 1°C/min (Fig. 3.18). Results indicated that oocytes freezing injury is more likely to be caused by the period of exposure at sub-zero temperatures. The protective effect of 4M methanol was much more pronounced when oocytes were cooled at 1°C/min showing that 1°C/min was more suitable for cryopreservation of stage I and II zebrafish oocytes when compared with 0.3°C and 0.5°C/min cooling rate (Figs 3.17-18).



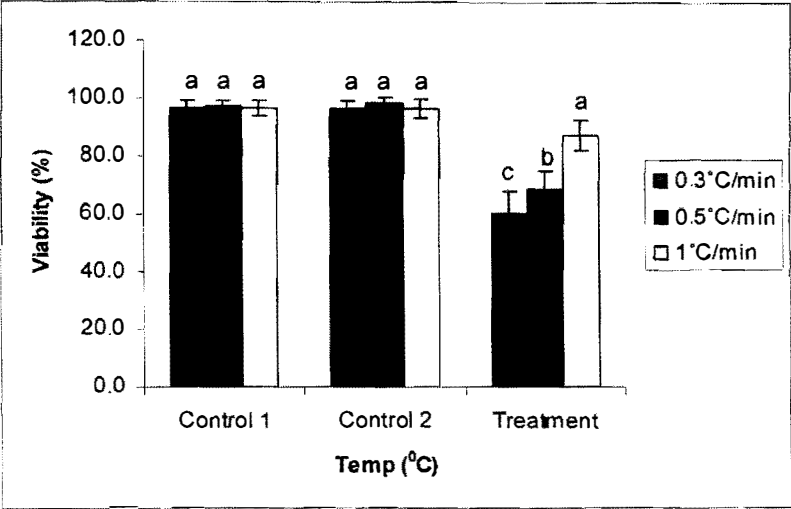


Figure 3.17. TB-assessed viabilities of stage I oocytes.

Oocytes were frozen to  $-196^{\circ}\text{C}$  in 4M methanol KCl-buffer at different cooling rates. Control 1 is the initial viability of oocytes in KCl-buffer assessed with TB staining. Control 2 is the viability of oocytes in KCl-buffer, incubated at  $22^{\circ}\text{C}$ , assessed with TB staining after 30min incubation at  $22^{\circ}\text{C}$  before cryopreservation.

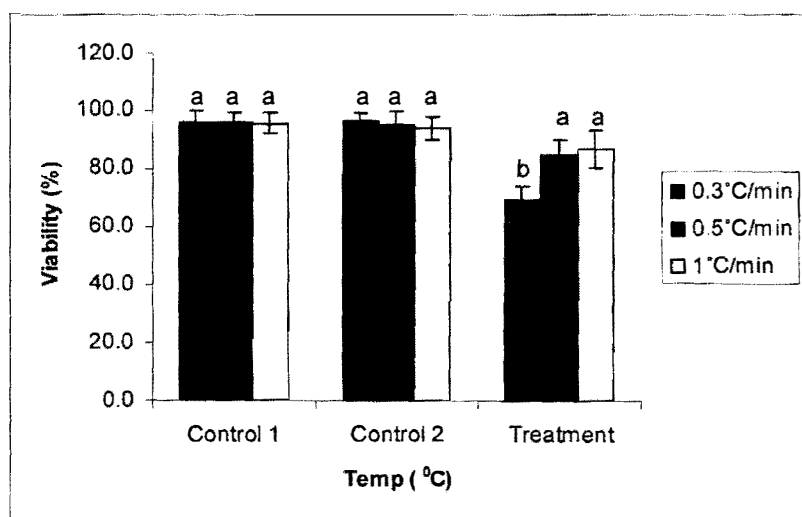


Figure 3.18. TB-assessed viabilities of stage II oocytes.

Oocytes were frozen to  $-196^{\circ}\text{C}$  in 4M methanol KCl-buffer at different cooling rates. Control 1 is the initial viability of oocytes in KCl-buffer assessed with TB staining. Control 2 is the viability of oocytes in KCl-buffer, incubated at  $22^{\circ}\text{C}$ , assessed with TB staining after 30min incubation at  $22^{\circ}\text{C}$  before cryopreservation.

### 3.3.1 Effect of incubation period after cryopreservation

In these experiments, cryopreserved oocytes after thawing and washing from cryoprotectant in one-step removal method used were incubated in KCl-buffer medium at room temperature for 60 and 120min and oocytes viability were then assessed using TB staining.

Stage I and II oocytes in the presence of 4M methanol solution were incubated for 30min at 22°C and then cooled at the rate of 1°C/min. Control survivals before cryopreservation (control 1) of stage I and II oocytes at room temperature were  $97.6 \pm 2.6\%$  and  $97.4 \pm 3.3\%$ . After cryopreservation and incubation in KCl-buffer medium for 60min at room temperature, there were significant differences between stage I and II oocyte survivals, which were  $89.6 \pm 3.2\%$  and  $93.7 \pm 5.3\%$ . After 120mins incubation, there were significant differences between oocyte survivals, which were  $88.59 \pm 2.6\%$  and  $93 \pm 2.1\%$  respectively. However, there were no significant differences in oocyte survivals immediately after cryopreservation for stage I and II, which were  $87.8 \pm 4.9\%$  and  $86.8 \pm 6.5\%$  respectively. The survivals of stage I oocytes were significantly lower than the survivals of stage II oocytes after freeze-thawing and incubation in KCl-buffer for 60 and 120min at 22°C (Fig. 3.19), although incubation time period, 60 or 120min had no significant effect on oocyte survivals for both stage I and stage II oocytes. TB staining revealed that oocyte survivals increased significantly if stage II oocytes were incubated in room temperature after freezing. Incubation increased oocytes viability to  $93.7 \pm 5.3\%$  or  $93 \pm 2.1\%$  for 60 or 120min compared with  $86.8 \pm 6.5\%$  obtained immediately after freezing for stage II oocytes respectively.

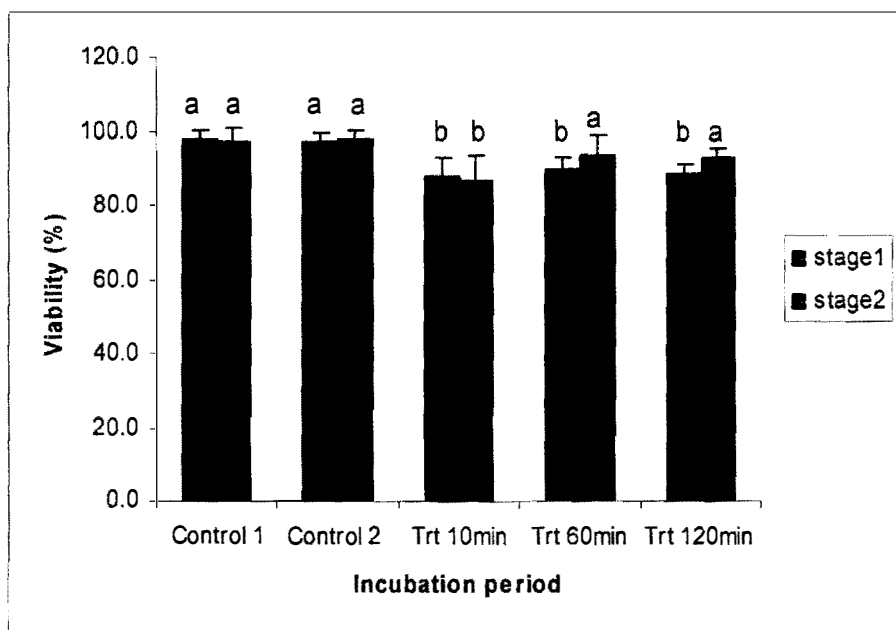


Figure 3.19. Effect of incubation on stage I and II zebrafish oocytes after cryopreservation and incubation at 22°C. Cooling rate was 1°C/min. Control 1 is the initial viability of oocytes in KCl-buffer assessed with TB staining. Control 2 is the viability of oocytes in KCl-buffer, incubated at 22°C, assessed with TB staining after 30min incubation at 22°C before cryopreservation.

## 4.0 Discussions

### 4.1 Toxicity studies on cryoprotectants

In this study, the effects of cryoprotectant toxicity to stage I and stage II zebrafish oocytes, have been reported for the first time. These oocytes are immature oocytes, they are 30% less hydrated than the mature oocytes (Wallace & Selman, 1981). Evidence have shown that immature fish oocytes are more permeable to solute than mature oocytes (Pennequin *et al*; 1975., Ecker & Smith, 1971, Seki *et al*; 2005, Smith & Ecker, 1969) due to their intact membrane channel protein. Trypan blue (TB) was used for evaluation of viability of zebrafish oocytes due to its fastest assay and can also be applied to oocytes at all developmental stages which allow the comparisons to be made. The results indicated that stage I (primary growth stage) oocytes, stage II (cortical alveoli stage) oocytes and stage III (vitellogenic stage) oocytes are less sensitive to methanol and DMSO when compared to PG and EG as shown in the NOEC in Table 3.1. The toxicity of cryoprotectants increased in order of methanol, DMSO, PG and EG. Methanol is the least toxic cryoprotectant to zebrafish oocytes when compared with DMSO, PG and EG.

Methanol has extremely high rate of permeation into various type of cells (Walsh *et al*; 2004, Lui *et al*; 2003, Ashwood Smith, 1987) and the relatively low toxicity of methanol to fish oocytes may be due to the easy penetration into cells and the activity of alcohol dehydrogenase (ADH) in oocytes which

oxidizes methanol (Rout & Armant, 2002). The present investigation identifies methanol as the least toxic cryoprotectant and with the previous studies by Plachinta *et al* 2004, which shows that the toxicity of methanol to oocytes at all developmental stages is lower than toxicity of the other tested cryoprotectants. Evidence has proved that methanol is relatively non-toxic for fish at the organism and cellular level, as supported by numerous animal exposure experiments ([http://www.epa.gov/chemfact/s\\_meth.txt](http://www.epa.gov/chemfact/s_meth.txt)).

DMSO is less toxic to fish oocytes, it has a distinctive property of penetrating into oocytes very readily. It does not need any protein channels to penetrate through plasmatic membrane and diffuses straight across phospholipid bilayer (Sum & de Pablo, 2003). PG was toxic when compared to methanol or DMSO, while EG was the most toxic as shown in Chapter 3 (Table 3.1). Zebrafish oocytes are very sensitive to EG at all stages. The high toxicity of EG to fish oocytes may be due to the relatively disabling effect on protein structure (Arakawa *et al*; 1990). Results from the present study showed that the effect of cryoprotectants on zebrafish oocytes increased with increasing concentration.

## 4.2 Cryopreservation of zebrafish oocytes using controlled slow cooling

There is harmful effect of cold on chromosomes and microtubules in human and other mammalian oocytes (Picton *et al*; 2000). Meiotic spindles are formed through the action of microtubules and are exquisitely sensitive to changes in temperature. It has been shown that cooling may induce irreversible disassembly of meiotic spindles (Wu *et al*; 1999, Zenze *et al*; 2001). Cold induces  $\text{Ca}^{2+}$  release followed by oocyte parthenogetic activation (Ben-Yosef *et al*; 1995, Zenze *et al*; 2001). Slow cooling cryopreservation protocols are protocols that involves cooling rates of  $10^{\circ}\text{C}/\text{min}$  or less. Although cryoprotectants in concentrations insufficient to vitrify protect cells that are cooled slowly, they offer no protection to cells cooled at excessively high cooling rates because water has insufficient time to diffuse out of the cell or is damaging cell membranes by the velocity of the osmotic diffusion. However cooling rates which are too slow expose the cells to toxicity of cryoprotectants and to solution effects (Mazur *et al*; 1984, Kalsson *et al*; 1996).

Stage I and II oocytes were used for slow cooling due to the relatively low sensitivity to the toxic effect of CPAs and permeability to water and solute. Three different cooling rates (0.3, 0.5 and  $1^{\circ}\text{C}/\text{min}$ ) were tested and the optimal post-seeding cooling rate for zebrafish was shown to be  $1^{\circ}\text{C}/\text{min}$  while  $0.3^{\circ}\text{C}/\text{min}$  provided the least survivals. This may be because  $0.3^{\circ}\text{C}/\text{min}$  slow cooling rate exposed the oocytes to the toxic action of CPAs.  $1^{\circ}\text{C}/\text{min}$  cooling

rate was shown to be more suitable than 0.3°C/min or 0.5°C/min and it is possible that relatively small size of stage I and II oocytes has a higher surface to volume ratio, therefore allowed faster cryoprotectant penetration and faster cooling rates. 1°C/min cooling rate, may have allowed sufficient time for cell dehydration therefore avoiding intracellular ice formation. The surface area to volume ratio for stage I, II and III were 1:3, 1:4 and 1:9 respectively when calculated. CPAs must be able to reach all parts of a cell quickly, and when volume is too low relative to surface area, diffusion cannot occur at sufficiently high rates to ensure cell survival.

These oocytes may have dehydrated relatively sufficiently to maintain the chemical potential of their intracellular solution. Other researches (Muldrew & Mc Gann, 1990) have demonstrated that the percentage of cells, which undergo IIF, was normally strongly dependent on cooling rate.

The present study demonstrated that recovery conditions affect oocyte survival after cooling. Oocytes which had been incubated and warmed up to room temperature for 60 and 120min showed higher survival rate than those assessed with TB staining immediately after freeze-thawing. Oocytes improvement in survival after warming may be due to the activation of oocyte repair systems. It is possible that the repair process may have taken some time to acquire effects and to restore normal physiological condition of oocytes after cooling. The one hour and two hours incubation of oocytes at room temperature may have been enough for oocytes to repair some of the damage to their membranes. This type of repair system has been shown in the studies of chilling sensitivity of zebrafish oocytes by Isayeva et al (2004).



Recovery conditions affect oocytes survival after chilling. Mechanisms of repair system was also shown for starfish and sea urchin oocytes by Terasaki *et al* (1997). The mechanism for this is believed to be  $\text{Ca}^{2+}$  dependent.  $\text{Ca}^{2+}$  causes rapid fusion of cytoplasmic organelles with one another to form new impermanent surface membrane barrier (Terasaki *et al* 1997). The result also indicated that stage II oocyte survivals were significantly higher than stage I oocytes after cryopreservation and incubation at room temperature (22°C) for 60 or 120min. This shows that stage I oocytes were more susceptible to cooling when compared to stage II oocytes. It is generally believed that early developmental stages are more susceptible to chilling injury than mature oocyte. It was shown that immature bovine oocytes were more sensitive to chilling than mature oocytes (Arav *et al*; 1996), GVBD-stage sheep oocytes were more sensitive to chilling than MI-stage oocytes (Moor & Crosby 1985) and vitellogenic oocytes (stage III) were more susceptible to chilling than mature oocytes (IV) (Isayeva *et al* 2004). The present results agreed with these works and indicate that stage I (primary growth stage) oocytes are more sensitive to cooling than stage II (cortical alveoli stage) oocytes. Stage II oocytes were found to be the optimal developmental stage for cryopreservation. These results have shown that stage II oocytes can be more favourable for cryopreservation when compared with stage I and III oocytes. For TB assessed viability with the optimal freezing protocol, the percentage of viable oocytes after cooling to -196°C for stage II oocytes was  $86.8 \pm 6.5\%$ , however, survival obtained with stage III oocytes was  $19.6 \pm 8\%$  under the same condition (Plachinta *et al*; 2004).

## 5.0 Conclusions

### 5.1 Summary of results

The effects of cryoprotectant toxicity in stage I, II and III zebrafish oocytes were investigated and the optimal cooling rate and one step remover of cryoprotectant was used. Four widely used penetrating CPAs namely, Propylene glycol (PG), Ethylene glycol (EG), Dimethyl Sulfoxide (DMSO) and methanol were used, and three slow cooling rates (0.3, 0.5 and 1°C/min) and 4M methanol were tested in this study. TB staining showed that cryoprotectant toxicity of oocytes at stage I, II and III increased in the order of methanol, DMSO, PG and EG. No Observed Effect Concentration (NOECs) for methanol, DMSO, PG and EG were >5M, >4M, 4M and 1M for both stage I and II oocytes respectively. Methanol was shown to be the least toxic cryoprotectant. Oocytes toxicity increase with developmental stage.

Results obtained in this study on cryopreservation using controlled slow cooling showed that 1°C/min was the optimal cooling rate for stage I and II oocytes. The survivals of stage I oocytes were significantly lower than the survival of stage II oocytes after incubation for 60 or 120min, although there were no significant differences between the two stages immediately after cryopreservation when tested with TB staining. TB staining revealed that oocyte survivals increased significantly if stage II oocytes were incubated in room temperature after freezing. Incubation increased oocytes viability to 89.6

$\pm 3.2\%$  and  $93.7 \pm 5.3\%$  or  $88.6 \pm 2.6\%$  and  $93 \pm 2.1\%$  for 60 or 120min compared to  $87.8 \pm 4.9\%$  and  $86.8 \pm 6.5\%$  obtained immediately after freezing for stage I and stage II oocytes respectively.

## 5.2 Conclusions

There were no significant difference observed between stage I and II oocyte survivals immediately after cryopreservation, although significant difference were observed between the two stages after incubation at room temperature for 60 or 120min. There were increase in their survivals after incubation, this shows that recovery conditions affect oocyte survival after freezing. However, it was found in this study that oocytes incubated for 60 or 120min have higher survival rate than those assessed immediately after cryopreservation.

Stage II oocyte survivals reported from the present study were significantly higher than stage I oocytes and previously reported results for stage III oocytes showing that stage II oocytes were more resistible to freezing than stage I and III oocytes and consequently most suitable stage for cryopreservation. High level of survival ( $89.6 \pm 3.2\%$  and  $93.7 \pm 5.3\%$ ) of stage II oocytes were obtained after 60min incubation using TB staining.

More work needs to be done in using other viability assessment e.g in vitro maturation followed by observation of germinal vesicle breakdown (GVBD), measurement of the ADP and ATP, fluorescein diacetate (FDA) and propidium iodide (PI), known as FDA+PI staining and the use of molecular

markers methods to validate these results, since TB staining can only assess the membrane damage as opposed to whole cell physiological status.

### 5.3 Future work

Inasmuch as stage II oocytes was proven to be the optimal developmental stage for cryopreservation when tested with TB staining, there is a need for the use of new viability assessment methods to validate these results.

*In vitro* maturation method for early stage oocytes also needs to be developed if these oocytes are to be of importance in future applications.

Vitrification have been developed and shown to provide a practical solution for cryopreservation of complex tissues that cannot be adequately preserved by freezing and thawing method. Cryobiological experience suggests, that many materials that cannot be successfully cryopreserved by controlled slow cooling may be cryopreserved by vitrification (Kaseoglu *et al*; 2001, Robles *et al*; 2005, Hamatoglu *et al*; 2005, Kopeika *et al*; 2006, Chen & Tian; 2005). Consequently vitrification should also be investigated in future experiments to reduce intracellular ice formation (IIF) in cryopreservation of fish oocytes.

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